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THE RELATION BETWEEN TYROSINASE AND ESTRINASE IN POTATO EXTRACTS[1]

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IT HAS BEEN demonstrated that liver and spleen (Zondek, 1934) and hyacinth root (v. Euler and Zondek, 1934) inactivate estrone in vitro. The inactivation cannot be due to an esterification of estrone since acid hydrolysis of the incubated mixtures liberates only a small part (6–20%) of the original estrone quantity. The effect is clearly enzymatic. The enzyme concerned was called estrinase (Zondek, 1935). Zondek (1941) demonstrated inactivation of estrone with the following plant materials: red beet, bran, and potatoes. Westerfeld (1940) demonstrated that preparations of mushroom tyrosinase inactivate estrone. He assumed inactivation to be caused by oxidation, the active hydroxyl group of C_3 in the estrone molecule being regarded as the site of oxidative inactivation in the body. Zondek and Sklow (1942) described an enzyme from potatoes which inactivated estrone. Since extract of mealworm larvae containing tyrosinase does not inactivate estrone, it was considered that estrinase is qualitatively different from tyrosinase. Graubard and Pincus (1942) found that mushroom laccase and potato tyrosinase oxidize and inactivate estrogens. Purified mushroom tyrosinase, however, failed to inactivate estrone. Graubard and Pincus expressed the opinion that this difference is due to a variation in tyrosinase substrate specificity from source to source.

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[1] In view of the difficulties of international communication the authors have not read proof of this article.

[2] Generously aided by a grant of the Rockefeller Foundation.

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In the present work, tyrosinase of potatoes was purified in order to examine the dependence of estrone inactivation upon either monophenol oxidase (cresolase) or poly-phenol oxidase (catecholase).

Experimental

The method of purification adopted was similar in general to the method of Kubowitz (1938) for the preparation of phenoloxidase from potatoes.

The potatoes were washed and the peel together with the external part of the potato was grated on a glass grater. One kg. of the resulting pulp was extracted with 500 cc. cold water. The mass was pressed through gauze, and centrifuged to give extract "a" (18–20 mg. dry matter per cc.). The extract was cooled and brought to 37 vol. % acetone. The sediment was discarded. To the filtrate further acetone to 55 vol. % was added. The precipitate obtained was taken up in 30 cc. of cold water, the insoluble residue being discarded. To the supernatant fluid, cold saturated solution of ammonium sulfate was added until 0.42 saturation was reached. After 3 hours at 5° C. the fluid was centrifuged. The precipitate was discarded, and to the supernatant more saturated $(NH_4)_2SO_4$ solution was added until 0.68 saturation was reached. The solution was allowed to stand for 12 hours at 5° C. The precipitate was packed by centrifugation and dissolved in 25 cc. cold water. The solution was dialyzed against running tap water for 24 hours, and the formed precipitate was centrifuged and discarded. The supernatant was brought with acetate buffer (m/10, pH = 4.8) to pH = 5.4 and acetone added to 34 vol. %. The precipitate which formed was separated and redissolved in 10 cc. m/15 phosphate buffer of pH = 7.3 (material "b"). The dry matter content of this solution after dialysis against distilled water was found to be 10 mg/cc. The original solution was heated for 4 minutes at 59–60° C. and then cooled in ice water. The precipitate which formed was centrifuged and washed with 2 cc. of cold water; the washing was added to the supernatant fluid. To 10–12 cc. of this fluid 2 cc. of glycerol and 1.5 cc. glycine buffer of pH = 10.2 was added. The mixture was then heated for 5 minutes at 48° C, then cooled in ice water and brought with 1.5 cc. n/10 acetic acid to pH = 5.0, and centrifuged. The precipitate was washed with 2 cc. cold water, centrifuged and the wash water added to the supernatant. The fluid was treated again at 0° C. with saturated ammonium sulfate until saturation of 0.45 was reached and the precipitate was taken up in 8 cc. m/15 Na_2HPO_4. The solution was dialyzed against running tap water for 72 hours. The brown precipitate formed was centrifuged. The supernatant (material "c") was the active principle employed. It contained 6 mg. dry matter per cc.

In testing for tyrosinase activity, p-cresol and catechol were used as substrates The activity was determined manometrically in the Barcroft-Warburg respirometer. In the main chamber there was placed 5 mg. of p-cresol resp. catechol dissolved in 2.5 cc. phosphate buffer m/15 of pH = 7.3; in the central well, 0.2 cc. KOH 10%; and in the sidearm, 0.5 cc. of the enzyme solution. The enzyme and substrate solutions were mixed after 10 minutes incubation in a water bath at 30° C. All enzymic preparations studied as to the estrinase content were tested for tyrosinase activity. It is inconvenient to express the enzyme activity on the basis of manometric measurements carried out on catechol, as the activity on this substrate falls off during the test increasingly as the enzyme is purified. The time curves on p-cresol showed

an initial induction period which increased in length as the enzyme purity was increased, and a later linear phase whose slope gives a convenient measure of the enzyme activity (Nelson and Dawson, 1944). The cresolase activities (mm^3O_2 2/5 min./10 mg. dry matter of enzyme) expressed on this basis were 12; 34 and 62.7, for materials a, b and c respectively. The recovery of enzyme in terms of cresolase activity is thus 1.4% in material b, and 1.2% in material c as compared to material a. Representative results are presented in Figure 1.

All these preparations were free from laccase as tested on p-phenylene diamine and guaiacol.

In testing for estrinase activity, an aqueous solution of estrone[3] containing 4 gamma of estrone per cc. was employed.[4] 2.5 cc. of this solution

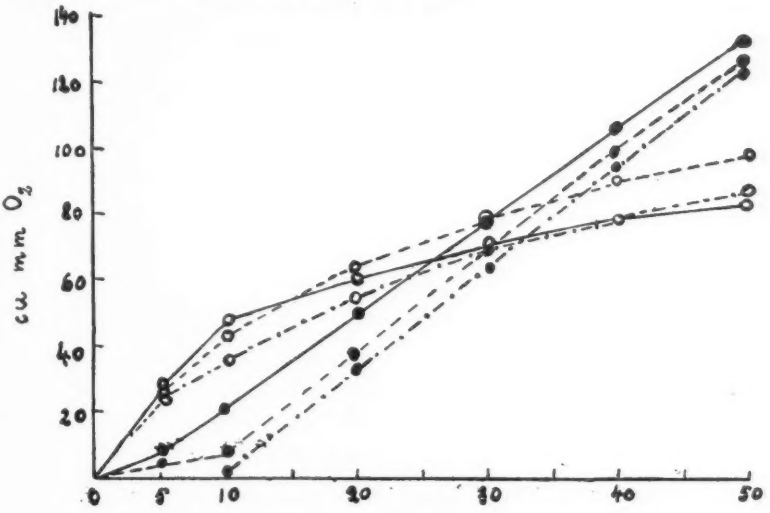

FIG. 1. Oxidation of p-cresol and catechol by tyrosinase from potatoes at different stages of purification process

Temperature 30° C.
● p-cresol (5 mg.) ○ catechol (5 mg.)
——— preparation "a"; - - - - preparation "b";
—·—·—preparation "c";

(containing 10 gamma of estrone) were put in a rocking incubator for 12 hours at 30° C. with 0.5 cc. of our potato tyrosinase extract and 1 cc. of phosphate buffer of pH = 7.3. The enzymic action was interrupted by heating the solution in a boiling water bath for 10 minutes. The estrone content was then determined by the Allen-Doisy test. For each experiment 5 to 25 castrated female mice were used, making up a total of 225 animals for all

[3] We are indebted to Dr. B. J. Brent, Roche-Organon, Nutley N. J., for the supply of estrone.

[4] To 0.1 cc. of an alcoholic solution containing 2 mg. estrone per 1 cc., 50 cc. distilled water was added and the solution was then heated at 80° C. till the alcohol was evaporated, the remainder being made up to 50 cc. with distilled water.

fractions studied (cf. Table I). Controls using heated enzyme with estrone solution were performed in every case.

RESULTS

The estrinase activity of the various preparations at 3 different stages of purification is given in Table I (preparations "a," "b," and "c"). The preparations "a" and "b" contain active estrinase, estrone being inactivated by these preparations to the extent of 90–100%.

On further purification the estrinase activity is lost, as is demonstrated by findings with material "c." Five preparations of "c" were found entirely lacking in estrinase; two preparations of "c" contained

TABLE I. ESTRINASE ACTIVITY AT DIFFERENT STAGES OF THE PURIFICATION PROCESS

Enzymic preparation	Serial no.	Number of animals used	Recovery of estrone %
a	1	10	0
	2	10	2
b	1	30	<10
	2	20	<10
	3	10	0
	4	10	0
	5	10	0
c	1	10	100
	2	20	100
	3	10	100
	4	5	60
	5	5	100
	6	10	60
	7	25	100
sweet potatoes extract	1	10	100

up to 40% of the original estrinase strength. Further precipitation in these two cases led to complete disappearance of the estrinase activity. The procedure employed thus effectively frees tyrosinase from the accompanying estrinase, but does not yield an estrinase free from tyrosinase. All fractions found to contain estrinase also gave tyrosinase effects. It is of interest that the estrinase activity does not diminish gradually with the successive steps of purification, but is eliminated sharply by the precipitation with ammonium sulfate in the preparation of material "c." We were unable to reactivate this fraction to estrone either by addition of crude potato extract boiled for 3 minutes or by addition of traces of catechol.

A crude extract from sweet potatoes which contains only catecholase but no cresolase was inactive towards estrone and could not be activated either by boiled crude extract of potatoes or by catechol. These findings are in agreement with those of Westerfeld (1940), who showed that catecholase and catecholase-catechol do not inactivate estrone.

DISCUSSION

The estrone inactivating system in crude potato extract is not identical with tyrosinase, cresolase (mono-phenol oxidase) or catecholase (poly-phenol oxidase), since purified potato extract although it retains cresolase and catecholase activity is inactive to estrone. This is in agreement with previous observations of Zondek and Sklow (1942), and Graubard and Pincus (1942), who found that tyrosinase from various sources (mealworm and mushroom) is unable to inactivate estrone. There is no difference in principle as regards estrone inactivation between the tyrosinases of mushroom, mealworm, and potatoes, but whereas mealworm tyrosinase fails even in the crude state to act on estrone, tyrosinase preparations from mushroom and potato only become inactive to estrone after purification.

Graubard and Pincus (1942) ascribed the estrone inactivating activity of the mushroom preparation of Westerfeld (1940) to the laccase present in Westerfeld's extract. Our potato extract, however, does not contain laccase and, hence, laccase could not be responsible for the estrone-inactivating effect of potato extract.

Thus it has so far been shown that

a) mushrooms contain tyrosinase (the mono-phenol oxidase: cresolase+the poly-phenol oxidase: catecholase) and laccase and estrinase, the latter action being due possibly to laccase:
b) mealworms contain tyrosinase but not estrinase:
c) potatoes contain tyrosinase, and estrinase, but not laccase.

These results show that laccase and estrinase activity are not necessarily linked. Hence laccase and estrinase may be regarded as distinct entities. Likewise tyrosinase activity is not necessarily accompanied by estrinase activity. Hence the estrinase system cannot be identical with tyrosinase. On the other hand, estrinase has not so far been obtained free from tyrosinase, though it has been found free from laccase. Only two explanations of the findings are compatible with all the facts:

1) that estrinase and tyrosinase are distinct and separate enzyme systems:
2) that estrinase is a system composed of tyrosinase plus a further heat labile factor.

Choice between these alternatives seems to be at present impossible. Explanation 2 is not intrinsically impossible since it is known that the substrate range of tyrosinase depends on the degree of the purification of the enzyme.

SUMMARY

A crude extract of potatoes which manifests estrinase and tyrosinase activity has been fractionated. A purified fraction contained tyrosinase but not estrinase. Active estrinase free from tyrosinase was not obtained. Addition of boiled crude extract of potatoes or traces

of catechol to purified tyrosinase did not restitute the estrinase activity originally manifested by the extract. Possible relations between tyrosinase and estrinase are discussed.

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THE EFFECTS OF ORAL AND PARENTERAL ADMINISTRATION OF A NEW SYNTHETIC ESTROGEN, BENZESTROL,[1] IN THE RAT

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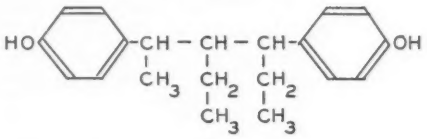
IN A PREVIOUS report the activity of certain members of a new series of synthetic chemical compounds exhibiting estrogenic activity was reported (Blanchard, Stuart, and Tallman, 1943). One of these, designated as Res. Labs. #118B, was sufficiently active to warrant further intensive study, since its potency was such as to make its clinical use feasible. We wish to report here the results of a series of studies undertaken to determine the effects of varying amounts of this compound, given by several routes of administration, upon the essential organs and systems in the rat. Preliminary reports of this work have been presented (Blanchard, 1942; Stebbins and Blanchard, 1942). The results of the study of the effect of the estrogen upon the hematopoietic system are being reported separately.

Many similar studies have been made upon the action of diethylstilbestrol, as have been summarized by Morrell (1941), and by the Council of Pharmacy and Chemistry (1942).

MATERIALS AND METHODS

It was the purpose of the study to compare the action of this new synthetic compound in all essential ways with the action of the natural steroid estrogens. The compound studied is one of the purified racemates of 2,4-di-(p-hydroxyphenyl)-3-ethyl hexane and exhibits the structure shown in Figure 1. It has a melting point of 161–162° C.



BENZESTROL

FIG. 1

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[1] 2,4-di(p-hydroxyphenyl)-3-ethyl hexane manufactured by Schieffelin & Co., New York City. Benzestrol has been recognized as the non-proprietary name of this compound (J.A.M.A., 126: 1085, 1944).

This compound gives a positive estrus response at 0.8–1.0 microgram when given in sesame oil by a single subcutaneous injection to castrate female rats (Long-Evans), weighing 150–200 gms.

In this study the new synthetic estrogen was compared in its action with a natural whole estrogenic hormone (Difco) obtained from pregnant mares' urine which was carefully standardized in terms of international units of estrone. All materials for parenteral use were made up in sesame oil, so calculated as to allow the test dose to be injected in 0.2 cc of oil. The estrogens to be given orally were made up in 40% ethyl alcohol. The usual dose, 0.5 cc, was administered by stomach tube. Benzestrol was also implanted subcutaneously in the form of fused pellets, weighing from 50–250 mg.

Inasmuch as it was planned to study the natural and synthetic estrogens at comparable levels based on estrogenic activity, an attempt was made to establish the ratio of activity between the two substances. In the case of the parenteral route of administration this was easily accomplished, and it was found that 0.8–1.0 μg. of the benzestrol produced the same estrogenic response as 2.0–2.5μg. of natural estrogen. When administered by the oral route it was more difficult to establish a ratio between the two estrogens because from week to week, the same groups of rats exhibited variations in response to both natural and synthetic estrogens. However, it was found that benzestrol is approximately 10 times more active by mouth than the natural estrogen.

In studying the effects of these estrogens, normal and castrate, young and adult female rats of the Long-Evans strain were used, and groups of at least six rats were used on each experimental level. The oral, parenteral, and pellet implantation studies continued for 12 weeks, and at least one rat from each group was sacrificed at the end of the 4th and 8th weeks. In some instances one or two of the animals were allowed to return to their pretreatment state and then studied to determine the extent to which the animals had recovered from the estrogen administration.

A careful examination was made of the gross appearance of all essential internal organs. Many of the organs were weighed and prepared for histological examination. A half of each pituitary was prepared for cytological study in order to determine the extent to which its secretory activity might have been affected.

EXPERIMENTAL RESULTS

That the synthetic estrogen under consideration would produce estrus in the castrate rat had been demonstrated (Blanchard, Stuart and Tallman, 1943). The mere fact that vaginal cornification followed the administration of the substance did not, in itself, prove that the substance would bring about the other changes characteristic of the natural estrogens. One such important property of the natural estrogens is the ability to prime the uterus so that the normal progestational endometrium will develop following the administration of progesterone. This can best be demonstrated in the castrate or immature rabbit. That benzestrol would bring about such a priming action was demonstrated as follows: To two of four castrate adult rabbits a total of 20 μg. of benzestrol was given over a period of six days, fol-

lowing which a total of 1 mg. of crystalline progesterone was given over five days. Laparotomy was performed 24 hours after the last progesterone injection, and a portion of the uterus taken and prepared for histological examination. To the other two castrate rabbits, 50 μg. of natural estrogen were given (500 I.U.), followed by progesterone and laparotomy as above. An examination of the two sets of sections showed that the synthetic substance primed the uterus in a manner qualitatively indistinguishable from the action of the natural estrogen.

Pituitary: Another characteristic action of natural estrogens is the ability to repair the changes which occur in the pituitary following oöphorectomy. Cytological examination of the pituitaries of castrate rats receiving benzestrol showed that the castration changes had been repaired: The large basophilic castration cells had disappeared, and in those rats receiving minimal doses, the pituitaries resembled closely the gland in the non-castrate rat. In those animals receiving large overdoses of benzestrol for a considerable period of time the pituitaries took on an acidophilic appearance which was identical with that shown by the glands following similar large and continued dosage of the natural estrogen. Following the cessation of treatment, the basophiles reappeared, the time of this reappearance correlating roughly with the amount, and the length of time, of the previous treatment.

In the normal, non-castrate female rat, continued therapy with the synthetic estrogen, as with natural hormone, also resulted in this acidophilic picture in the anterior pituitary. In those normal rats which were carried along for some time after the cessation of therapy the pituitaries were undoubtedly functioning normally as was evidenced by the return of the normal estrous cycle in these animals in about 10–14 days.

No atypical growth was observed in any of these pituitaries and in only 4 cases, equally divided between those animals receiving benzestrol and those receiving natural estrogen, was any appreciable hyperemia or sinus congestion seen.

It would seem to be quite evident that the long continued administration of this synthetic estrogen produced no changes in the pituitary gland other than those characteristic of normal estrogen overdosage, and that no permanent damage to the organ occurred. The pituitary returned to normal, as evidenced by a reëstablishment of the estrus cycle, as well as by cytological examination.

Organ Weights: In connection with the study of the possible toxic reactions of this synthetic estrogen, the weights of certain essential organs were determined. These included the several endocrine glands which might have been directly or indirectly influenced by the estrogenic activity of the synthetic compound, as well as those organs—the liver, spleen, and kidney—which might reflect any toxic properties of the benzestrol, quite apart from its action as an estrogen. While the

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adult rats used were reasonably uniform in weight, about 150–200 grams, the organ weights have been corrected to a body weight of 175 grams. The organ weights of the benzestrol treated rats were compared with those receiving comparable estrogenic doses of natural estrogen, and with untreated controls. Such data, as well as the results from pellet implantations, are shown in Table 1.

TABLE 1. AVERAGE ORGAN WEIGHTS IN GRAMS OF ADULT NORMAL FEMALE RATS RECEIVING BENZESTROL AND NATURAL ESTROGEN, AND OF CONTROLS

Organ	Length of treatment — days	Substance injected, dose level, and mode of administration. Each value represents the average of 6 animals.						Normal, untreated controls. Average of 24 animals
		Natural estrogen inj. 60 μg./wk.	Benzestrol inj. 30 μg./wk.	Natural estrogen fed 1500 μg./wk.	Benzestrol fed 1500 μg./wk.	Benzestrol 50 mg. pellets implanted	Benzestrol 250 mg. pellets implanted	
Pituitary	28	.008	.007	.009	.008	—	—	.008
	84	.010	.009	.008	.008	.009	.012	
Thyroid	28	.015	.015	.017	.018	—	—	.014
	84	.013	.019	.018	.017	.012	.013	
Liver	28	7.07	7.68	7.55	7.40	—	—	7.70
	84	8.94	8.02	6.90	7.10	7.35	7.60	
Spleen	28	.808	.680	.720	.825	—	—	.830
	84	1.300	.677	.680	.760	.860	.790	
Kidney	28	1.490	1.300	1.350	1.420	—	—	1.430
	84	1.430	1.321	1.390	1.290	1.400	1.350	
Adrenal	28	.066	.064	.048	.045	—	—	.055
	84	.080	.086	.050	.049	.080	.075	

As can be seen from this table, no significant difference in weight of any tissue or organ can be noticed between those rats receiving benzestrol, those receiving comparable amounts of natural estrogen, and the controls.

HISTOLOGICAL FINDINGS

Particular care was exercised in the examination of the several tissues for changes which might indicate toxic reactions to the synthetic estrogen. Controls were set up, using a like number of animals, receiving estrogenic equivalent amounts of natural estrogen.

No evidence of "toxicity" following long term administration of benzestrol was seen in any of the key structures studied. Certain changes from the normal were observed, as will be noted, but these changes were duplicated in every instance by like changes, qualitatively, occurring in the animals receiving natural estrogen. Therefore, it is concluded that such changes are due to the estrogenic activity of the compound acting either directly or through the pituitary, upon the other tissue. These changes with benzestrol and natural estrogen administration were practically identical with those reported by Morrell and Hart (1941) as following natural and synthetic estrogen administration.

Vaginal cornification was constant in the castrate females but the normal females showed periods during which mucification occurred. The uteri of both normal and castrate rats showed varying degrees of

estrogen action and a few local areas of metaplasia were seen. The uteri of normal rats which were carried for some time following cessation of treatment and in whom an estrous rhythm had been reëstablished appeared perfectly normal, indicating that no permanent change had occurred.

Ovaries: Some degree of depression of follicular development in non-castrate females at all levels of estrogen administration was noted by the end of the 4th week. This occurred with both benzestrol and natural estrogen, seemed to bear little quantitative relationship to the dosage used, and was reversible following cessation of estrogen administration.

Adrenals: There was no marked change in the adrenals, although there was some slight tendency toward cortical hypertrophy in all groups. There was slight hemorrhage in the cortical zones of the adrenals of 5 animals; 2 of these were receiving benzestrol, 30 micrograms per week; 2 receiving natural estrogen, 60 micrograms per week; and one receiving 3 mg. per week of natural estrogen orally.

Liver: Considerable concern has been felt over the possibility of liver damage following long continued synthetic estrogen administration. No evidence of serious liver damage could be found in any of the animals, irrespective of type of estrogen or dosage. There was some indication of cloudy swelling and fatty infiltration in a few instances, but these conditions have been found in normal animals and in animals receiving sesame oil alone. No difference in the liver picture was seen in those animals receiving estrogen orally, from those receiving parenteral administration. Such changes as occurred may be considered reversible, as the livers of rats allowed to live following treatment could not be distinguished from the normal controls.

Kidney: Nothing which could be regarded as serious kidney damage was observed. In a few instances there seemed to be a slight shrinkage of the tubules and glomeruli. In one case, a slight hemorrhage in the glomerular zone was observed in a rat receiving 60 micrograms of natural estrogen per week. These noted variations were probably without particular significance, and the kidneys of rats some time after cessation of treatment appeared perfectly normal.

Thyroid: Regardless of the dosage, or estrogen used, we were unable to find the degree of thyroid damage reported by Morrell and Hart (1941) for stilbestrol and natural estrogen. In all instances the thyroid retained its acinar structure and in no case was there marked leucocytic infiltration or fibrosis, although a slight, to moderate, leucocytic infiltration was observed in several thyroids. The presence and degree of these changes and state of glandular activity bore no relationship to the amount or length of dosage of the estrogen used. The majority of the thyroids examined appeared to be in the resting condition with moderate storage of colloid, but a few seemed to show definite activity. The thyroids of the non-castrate animals that had

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returned to the pre-treatment condition seemed to be completely normal. The acini were distinct. Leucocytic infiltration—if it was assumed to have occurred in some degree during treatment—had disappeared, and the gland gave the appearance of the normal functioning thyroid of the adult female rat.

EFFECTS UPON GENERAL METABOLISM

It is generally accepted that high estrogen dosage will result in the slowing of the rate of weight gain in young rats, and may bring about a

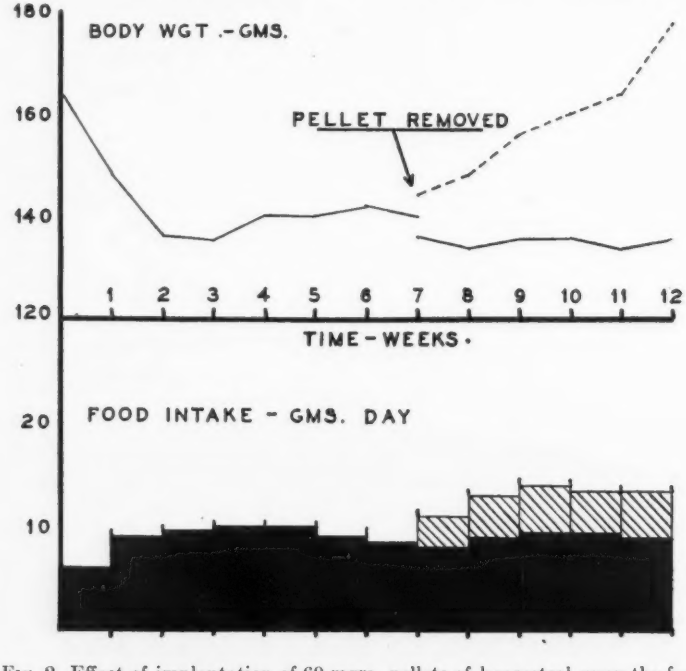

FIG. 2. Effect of implantation of 60 mgm. pellets of benzestrol upon the food intake and body weight. Broken line and cross hatched areas show increase in body weight and food intake in those rats from which the estrogen was removed at the end of the 7th week.

weight loss in adult animals. The mechanism by which this alteration is brought about is not well understood, but it seems that one of the factors is loss of appetite resulting in lowered food intake and consequent lowering in weight increment.

Three sets of data may be cited to show that benzestrol administration results in the expected effects upon body weight. In one series of rats, weighing at the start of the experiment 160 gms., the synthetic estrogen was administered by injection in oil for 70 days at a level of 60 micrograms per week. The average daily weight gain for

this group of 12 animals was 0.5 gms. per day. In a second series of 12 animals, weighing 120 gms., benzestrol was administered by mouth at a level of 300 micrograms per week. The average daily weight gain for the 70-day period was 0.75 gms. The average weight gain for normal untreated rats of this strain in this laboratory, in these weight ranges, is 2.0–2.5 gms. per day (Blanchard and Tallman, 1938). No record of the food intake was kept in the above series.

In Figure 2 is shown the effect of benzestrol pellet implantation in a group of 12 normal rats. The average weight of the pellets was 60 mgms., and the average daily estrogen absorption was 300 micrograms. In this series, daily food intake and body weight changes were recorded. As can be seen from the figures, considerable weight loss occurred. This seems to take place during the first three weeks with stabilization at a lower level. The food intake is shown in the lower part of the chart, calculated on a daily basis for each week. The average daily food intake for the first 7 weeks of experiment was about 9 grams per day. It was considerably lower than the average daily food intake (12–14 grams) for normal rats of this weight. At the end of the 7th week, the pellets were removed from half of the animals and the data collected for the next 5 weeks. As can be seen, the animals from which the pellets had been removed showed increased food consumption and began to gain weight. The group in which the pellets were left continued at the low food intake level and showed no weight increase.

No effort will be made to theorize as to the various factors which may interact to bring about the anorexia and weight loss.

A typical growth: Care was taken, in both gross and histological examination of all tissues, to look for evidences of atypical growths which might be considered malignant. Such examination was especially rigorous in those animals in which pellets had been implanted, particularly at the implantation site, where long continued direct contact between subcutaneous tissues and crystalline benzestrol had occurred. No change in tissue structure toward that of malignancy was found.

SUMMARY AND CONCLUSIONS

The synthetic estrogen benzestrol, 2,4-di(p-hydroxyphenyl)-3-ethyl hexane, was compared with natural estrogenic hormone at several dosage levels and by several routes of administration in normal and castrate, young and adult rats, and in castrate adult female rabbits.

A study of the physiological responses, and weight and histological changes of essential organs, showed that the action of this synthetic compound was identical in all respects with that of natural estrogens, and that no "toxicity" was observed from long continued overdosage. No evidence of malignant changes in any tissues was observed.

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THE EFFECTS OF A NEW SYNTHETIC ESTROGEN, BENZESTROL,[1] UPON THE HEMATOPOIETIC SYSTEM IN THE RAT

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From Research Laboratories, Schieffelin & Co.
NEW YORK, NEW YORK

THE NEW synthetic estrogen, 2,4-di(p-hydroxyphenyl)-3-ethyl hexane (benzestrol) has been subjected to intensive physiological investigation in this laboratory for the past three years (Blanchard, 1942; Stebbins & Blanchard, 1942). In our hands this compound produces the same type of estrus following a single subcutaneous injection in oil in the castrate rat at 0.8 to 1.0 micrograms as 2 to 2.5 micrograms of estrone (Blanchard, Stuart and Tallman, 1943).

In the toxicity studies of benzestrol the drug has been administered in sesame oil subcutaneously, in 40% alcohol orally by stomach tube, and by subcutaneous implantation of pellets.

METHODS AND RESULTS

Mature, normal and castrate female rats (120–180 gms.) of the Long-Evans strain were used in these studies. Oöphorectomized females were allowed a 2-week period of recovery, during which time daily vaginal smears were examined to confirm castration. R.B.C., W.B.C. (both G.S. pipettes), hemoglobin determinations (Hellige) and reticulocyte counts (wet method) were carried out at frequent intervals.

Preliminary determinations were carried out on 6 groups of 6 mature female animals each. Group I & II (castrates) received respectively 6 & 60 micrograms of natural estrogen[3] per week by injection; groups III & IV (castrates) received respectively 3 & 30 micrograms of benzestrol per week, by injection; groups V & VI

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[1] 2,4-di(parahydroxyphenyl)-3-ethyl hexane manufactured by Schieffelin & Co., New York City. Benzestrol has been recognized as the non-proprietary name of this compound (J.A.M.A., 126, 1085, 1944).

[2] A portion of this work was submitted by Robert B. Stebbins to the Department of Biology, Washington Square College, New York University in partial fulfillment for the degree of Master of Science. This author wishes to acknowledge the cooperation and advice given him by Drs. A. S. Gordon, H. A. Charipper and R. Gaunt during the course of this study.

[3] The natural estrogen used throughout this work was a whole natural estrogen material (Difco Laboratories) assaying 10,000,000 I.U. per gram. It is a mixture of ketonic and non-ketonic estrogens in unknown proportions.

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(normals) received respectively 3 milligrams of natural estrogen and 3 milligrams of benzestrol per week, orally. Many of these animals were sacrificed at intervals in order to examine organ and bone marrow changes. Therefore, some of the rats whose red blood cell counts were

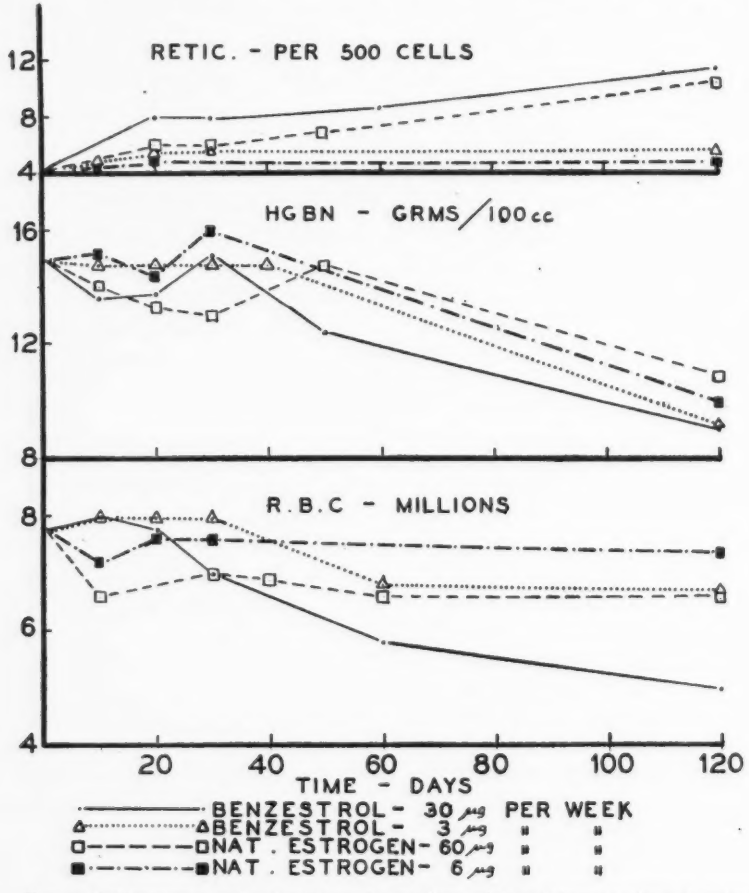

FIG. 1. Reticulocyte and red cell count, and hemoglobin determinations in rats receiving benzestrol (3 μg. and 30 μg. per week) and natural estrogen (6 μg. and 60 μg. per week) by subcutaneous injection.

included at the start of the experiment do not appear as part of the averages at the end of the study (see Fig. 1). Hemoglobin values parallel red blood cell changes and in the latter part of the experiment reticulocyte numbers increased slightly. Examination of the bone marrow of animals from this group showed erythroid hypoplasia with increased myelogenic areas. A tendency toward initial leucocytosis is

evidenced in these animals followed by inconstant fluctuations in white blood cells. Differential W.B.C. counts show no significant changes. The natural estrogen served as a control in this experiment and it is evident that benzestrol produces essentially the same response (Fig. 1). However, the drop in the red blood cells is not so

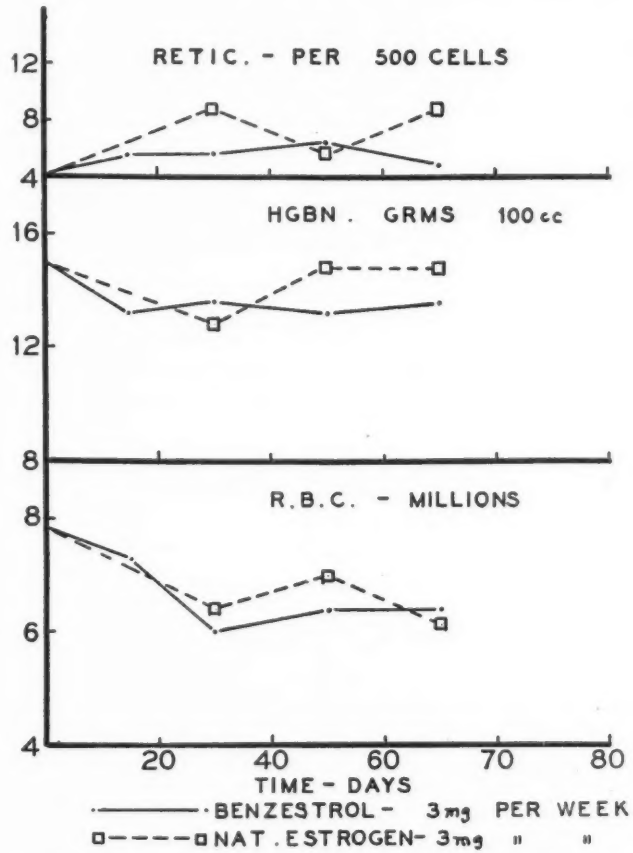

FIG. 2. Reticulocyte and red cell count, and hemoglobin determinations in rats receiving benzestrol and natural estrogen (3 mg. per week) orally.

pronounced with the natural estrogen. The effect following the injection of the two lower levels of benzestrol and natural estrogen appear in Figure 1. Benzestrol produces a slightly lower red blood cell response than the natural estrogen. Bone marrows from this group show mild hypoplasia with increased cellularity. Those animals which received benzestrol and the natural substance orally showed

varying decreases in erythrocyte number and percentage hemoglobin (Fig. 2). The decreases were appreciably less in these rats than in those receiving the material parenterally, and a marked tendency to return to the normal level during treatment was noted. Bone marrows taken at intervals from this group showed an increase in cellularity of the myelogenic elements followed by a general hypoplasia.

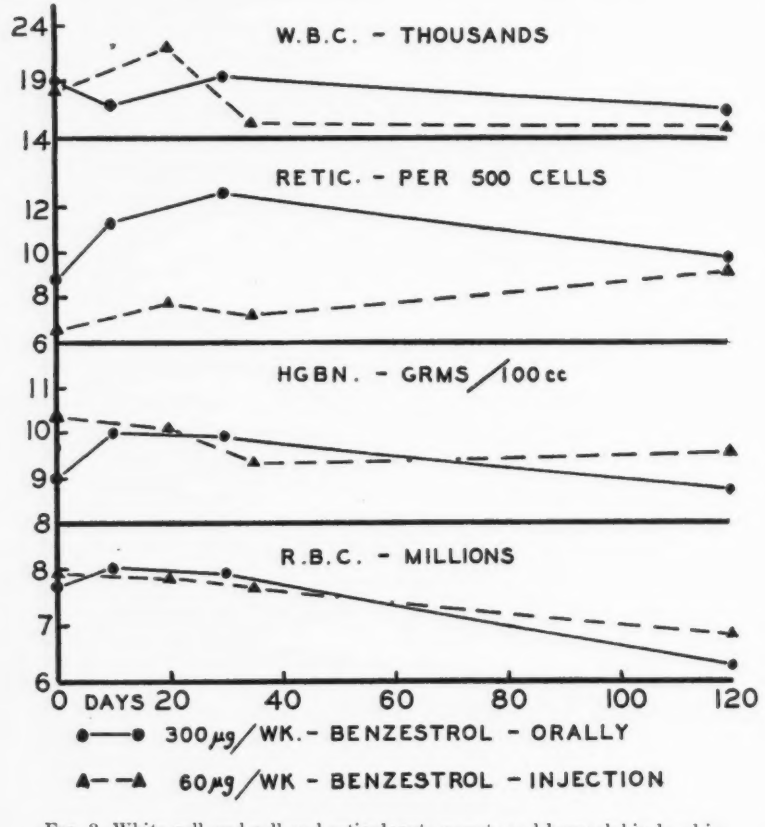

FIG. 3. White cell, red cell and reticulocyte count, and hemoglobin level in rats receiving benzestrol parenterally and orally.

A second and more extensive study was made upon two groups of normal female rats treated with large overdoses of benzestrol. One group received 60 micrograms of benzestrol by subcutaneous injection; a second group was given 300 micrograms, orally. Emphasis was placed upon the effects of benzestrol upon the W.B.C. system. As can readily be seen (Fig. 3), there is no tendency toward leucopenia in these experiments extending over a period of 120 days. The injected

animals exhibited a stabilization of W.B.C. at a level but slightly below the initial reading and within normal limits, while the orally-treated animals showed no noticeable change. In humans, these doses represent 30 milligrams per week and 150 milligrams per week parenterally and orally. As to the specific effects upon the numbers of erythrocytes, both groups showed a gradual decline in red blood cells with most of the decrease between the second and the fourth months. Nevertheless, there was only a decrease of slightly over a million cells in both the injected and oral groups during a 4-month period of treatment. None of these animals died and none were sacrificed as in the preliminary studies. No animal showed roughened fur, conjunctival hemorrhages, bloody diarrhea or vaginal bleeding as

TABLE I. DIFFERENTIAL COUNTS IN 24 ANIMALS RECEIVING BENZESTROL FOR 16 WEEKS

Average cell counts	Neutrophiles		Lymphocytes		Monocytes	Eosinophiles	Basophiles	Embryonic cells
	Non-fil.	Fil.	Large	Small				
Before treatment	1.0	13.7	25.6	53.2	3.5	2.9	0	0
After 16 weeks administration	9.9	12.5	23.3	43.4	3.75	3.1	1	3.3

reported for diethylstilbestrol (von Haam et al., 1941). Reticulocyte numbers and percentages of hemoglobin remained within normal limits throughout. Careful differential counts performed at the start of the experiment and at autospy on all of the above animals show a slight increase in non-filamentous neutrophiles and a slight decrease in small lymphocytes. Three animals showed increases in embryonic circulating cells after 4 months of continuous administration of the drug.

A third group of 28 mature animals received implantation of pellets as follows:—6 castrate females (51–61 mgm. pellets) and 2 castrate females (222–226 mgm. pellets); 6 normal females (52–61 mgm. pellets) and 2 normal females (213–250 mgm. pellets); 6 castrate females (108–119 mgm. pellets). Careful suturing insured against sloughing of the pellets. During this experiment certain animals were selected from each group and were reoperated for removal of their pellets, while others were allowed to continue indefinitely. Upon removal, the pellets were dried and weighed accurately to determine the average daily absorption (Table II). Each pellet upon removal was found to possess a strong fibrous capsule. Whitish cellular debris was occasionally discovered inside the capsule. No bacteria were found in this material. There was no disintegration or fracture of the pellets during the period of implantation and absorption appeared to be from the surface of the pellets.

In no case is there a significant leucopenia. White blood cell determinations on these animals show a tendency toward an initial

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stimulation with a gradual leveling off after continued absorption of the estrogen. Erythrocyte numbers again show a uniform decline. However, upon removal of the pellets there is a definite recovery of the red blood counts. This argues well for bone marrow rehabilitation. Hemoglobin percentages follow the general trend of the erythrocyte changes while the reticulocyte numbers increase with a decline of red blood cells, again indicating that the regenerating power of the bone marrow is not impaired. Histological examination of the bone

TABLE II. AVERAGE ABSORPTION OF BENZESTROL FROM IMPLANTED PELLETS

Groups	Pellet weight range	No. of rats	Av. days	Av. amt. benzestrol absorbed	Av. daily absorption
A	(51– 61 mg.)	6	45.8	16.0 mg.	349.3 μg.
A_1	(222–266 mg.)	2	53.5	52.0 mg.	971.9 μg.
B	(52– 61 mg.)	6	41.8	15.0 mg.	358.8 μg.
B_1	(213–250 mg.)	2	66.5	74.0 mg.	1112.7 μg.
C	(24– 31 mg.)	6	97.0	16.0 mg.	164.9 μg.
D	(108–119 mg.)	6	94.0	59.0 mg.	627.6 μg.

marrow showed, without exception, a highly cellular marrow which later reverted to one of hypoplasia and vacuolization upon continued therapy. This hypoplasia was completely repaired upon cessation of estrogen absorption. Thus it must be assumed that the initial effect upon the hematopoietic system takes place without overly excessive doses of the drug. However, there is a gradual decrease in red blood cells which is followed by a definite bone marrow response.

CONCLUSIONS

The experimental animals received large amounts of benzestrol by injection, implantation and by mouth without manifestation of any untoward symptoms. Most of these animals showed a lowering of their red blood cell counts but this was always followed by an output of reticulocytes and a stabilization of erythrocytes at a slightly lower level than normal. In no case was there a depression of hematopoiesis. Considering the excessive dosages used in these experiments, benzestrol, when used within therapeutic range, would seem to be non-toxic to the hematopoietic system. Both benzestrol and the natural estrogen produced less change when administered orally than when given parenterally.

In the implantation experiments a large amount of the estrogen was absorbed, yet these same animals did not suffer irreparable damage to their hematopoietic systems. In those animals where the pellets were removed after comparatively long periods of time, the bone marrow and other hematopoietic centers again produced the formed elements. There is no significant tendency toward a leu-

copenia in any of the experimental animals. Other than an initial stimulation of white blood cells upon administration of benzestrol, and an occasional animal showing some embryonic circulating cells after long continued treatment, there is nothing to suggest an upset of the leucocytic series of cells. The histology of the bone marrows of all the treated animals show evidence of hypoplasia with natural estrogen as well as benzestrol. Many of the long term pellet implantations show some vacuolization, but upon removal of the pellet this is completely repaired. There can be no doubt as to the regeneration of the bone marrows of these treated animals. Reticulocyte and hemoglobin changes follow closely the trend of the red blood cell reaction to the estrogen. Reticulocytes tend to show a gain with a fall in red blood cells, whereas hemoglobin values tend to follow the changes in red blood cell count. Of the six groups of pellet-implanted animals there was not one which maintained its weight to the end of the experimental period. This is not in complete accord with the results reported by Page and co-workers (1941), for diethylstilbestrol. They report normal animals implanted with 50 mg. pellets showed a consistent weight gain, while other similarly treated animals lost weight.

Our animals (castrate and normal) in the 200 mg. pellet class showed very little variation in over-all absorption. By and large, there appears to be a definite relationship in the amount of absorption between the groups (whether castrate or normal) dependent upon the size of the pellet. Inasmuch as absorption in castrate and normal groups from similar size pellets was approximately the same, it may be assumed that the rate of absorption is not conditioned by the estrogen need of the animal.

SUMMARY

The effects of physiologically equal doses of benzestrol and natural estrogen were studied in mature normal and castrate female rats. The administration, both orally and parenterally, of benzestrol in doses from 3 micrograms to 3 milligrams per week produced an effect on the formed elements of the blood and bone marrow of rats qualitatively indistinguishable from that of the natural estrogen. The temporary effects observed are somewhat more striking from 3 and 30 micrograms of benzestrol than from 6 to 60 micrograms of natural estrogen.

The animals that received benzestrol in pellet form showed red blood cell counts which were below normal. Upon removal of the pellet these counts returned to the normal level. There was no evidence of leucopenia or continued depression of hematopoiesis.

From the results of these experiments it is evident that long continued administration of greatly excessive amounts of benzestrol has no permanently detrimental effect upon the hematopoietic system in rats.

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INFLUENCE OF ESTRADIOL AND TESTOSTERONE PROPIONATES ON SKELETAL ATROPHY FROM DISUSE AND ON NORMAL BONES OF MATURE RATS[1]

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IT IS A common clinical experience to observe an atrophy of the bones of an extremity which has been paralyzed for a considerable time. This form of atrophy from disuse also occurs in bones which, although not injured, are immobilized incidental to the treatment of a fracture of another bone. The degree of the atrophy may be marked since its presence is frequently demonstrable by ordinary roentgenograms. Allison and Brooks (1921) produced atrophic changes of the bones of dogs by section of nerves, or by excision of the upper end of the humerus. Using roentgenographic and histologic techniques, they observed the gross and minute changes that occurred in the atrophic bones. Two animals were employed to demonstrate changes in chemical composition of the humeri after 24 and after 256 days of brachial paralysis. It appears, therefore, that the atrophy of bone from disuse has not been adequately investigated, especially as to the degree of atrophy which occurs in a given time or as to possible alterations in the constitution of the atrophic bone. There is also no evidence in the literature as to whether the degree of bony atrophy is affected by nutritional alterations or by other systematically operating metabolic factors such as hormonal influences.

No simple method, which gives results in quantitative terms, has been described for the investigation of the healing of fractures. In this connection, a study of the factors which influence skeletal atrophy has a possible interest not related to bony atrophy *per se*. If the degree of atrophy of the bones of paralyzed limbs is susceptible of modification, and if it could be shown that the atrophy is a result of a decreased rate of formation of bone (rather than an increased rate of resorption of bone), a simple procedure would then be available for the discovery and comparison of factors of probable influence on the

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[1] This work was initiated under the terms of a contract, recommended by the Committee on Medical Research, between the Regents of the University of Minnesota and the Office of Scientific Research and Development. It was completed under support of a grant from the Josiah Macy, Jr. Foundation.

healing of fractures. Therapy based upon these factors might have a rational use in the treatment of fractures as adjuncts to the surgical methods of reduction and fixation. The mechanism of the atrophy occurring in the bones of paralyzed limbs is being investigated, as is also the effects of nutritional variations on the degree of atrophy (Armstrong, 1945).

The present investigation was undertaken primarily to determine whether the degree of disuse atrophy of bones is affected by certain sex hormones. A by-product of this study is an addition to the information in the literature relative to the effect of these hormones on normal bones of mature animals. It seemed possible that some of the sex hormones might affect the atrophy of bones since castration and the administration of estrogenic and androgenic hormones are known to influence epiphyseal growth, medullary calcification and the composition of bones of normal limbs to degrees which vary qualitatively and quantitatively with the species investigated and with the age and sex of the animals (Gardner and Pfeiffer, 1943). Among mammals the most striking effects are reported to occur in the bones of mice treated with large doses of estrogens. The medullary cavity of the femurs becomes hyperossified, the ash content of the bones is increased and the calcium to phosphorus ratio of the bone ash is increased (Wentworth, Smith and Gardner, 1940). Day and Follis (1941) found somewhat similar, but less marked effects on the structure and percentage of ash in the femurs of young or young adult rats treated with estradiol benzoate for 44 days. However, the bones of older rats (133 to 650 days of age at the beginning of treatment), while exhibiting some microscopic evidence of increased trabeculation, showed no significant increase in ash content.

A variety of opposing effects on skeletal growth have been reported to result from the administration of androgens to immature laboratory rodents (Gardner and Pfeiffer, 1943). Little effect on the percentage of ash or on the calcium to phosphorus ratios of the femurs resulted from treatment of mice with testosterone propionate beginning at an early age and continuing over long periods of time (Wentworth, Smith and Gardner, 1940). The length of the femora and humeri of rats and their density as judged from roentgenograms was not affected by treating young rats with the same hormone for 87 days (Turner, Lachmann and Hellbaum, 1941).

METHODS

In mature male rats of the Sprague-Dawley strain, certain nerves from the cords of the brachial plexus were severed on one side to permit the development of disuse atrophy of one humerus.[2] Using aseptic technique, the

[2] It is recognized that disuse atrophy occurring in the bones of a paralyzed limb may not be identical in all aspects with that occurring in the skeleton of a limb immobilized in a cast or by skeletal fixation.

median, radial, ulnar, musculocutaneous, subscapular, axillary, thoracodorsal and suprascapular nerves (Greene, 1935) were exposed by splitting the pectoralis major muscle with a hemostat and these nerves were severed as they passed through the axilla. The operations were alternated between the right and left sides of the several animals in each group.[3] The animals were fed *ad libitum* on a diet of crushed commercial dog biscuit and were allowed to live 21 days after the operation. Table I indicates the nature of the experimental treatment of the several groups. The hormones were given by subcutaneous injections which were begun on the day of operation. In order to prevent the animals from chewing the paralyzed extremity, it was necessary to keep the incisor teeth cut short. The animals were sacrificed and the humeri, after prolonged boiling of the fore-limbs in water, were dissected free of soft tissue. The bones were rendered dry and fat-free by continuous extraction in a Soxhlet apparatus for 48 hours with an equal part mixture of alcohol and ether. After the bones were weighed, they were ashed to constant weight in platinum dishes in a muffle furnace at 700°.

RESULTS AND DISCUSSION

The mean initial and final body weights of the animals and the mean percentages of ash found in the humeri of the normal and paralyzed limbs are given in Table I. Table II is a presentation of the mean total dry, fat-free and total ash weights of the humeri, each expressed per 100 grams in initial body weight of the animals. The significance ratios shown in these tables are a measure of the probability of a statistically significant difference of the indicated mean from the corresponding mean of the controls (Group A).[4]

Effect of orchidectomy and of testosterone propionate on percentages of ash of humeri.—The data in Table I indicate that orchidectomy significantly reduced the ash content of both humeri below those of the untreated group (Group B). The same result was obtained in Group C which received 5 mg. testosterone propionate each day. A result in the same direction was secured in Group D. However, in this group the ash content of the humeri of the normally innervated limbs was barely at the border line of statistically significant difference from Group A, and in the case of the humeri of the paralyzed limbs

[3] It now appears that the alteration of the operations between the right and left sides of the animals was an unnecessary precaution since it has been found that there are no significant differences of weight or of ash weight of the two humeri of normal rats. The mean total ash weights, per 100 grams of body weight, of the right and left humeri of 20 unoperated rats fed a diet different from and probably superior to that employed in this study were respectively 55.58 ± 0.805 and 55.51 ± 0.583 mgs. These observations are in agreement with those of Weakley and Dustman (1939).

[4] The significance ratios involving means of Groups B, C, and G were calculated by dividing the difference of means to be tested by the standard error of the difference of means. In all cases involving means of Groups D, F, and H, Fisher's "t" test was employed because of the smaller number of animals in these groups. In the case of these latter groups the value of "t" is shown in the appropriate places in the tables. A difference of means is usually considered statistically significant if the significance ratio is 2.0 or greater than 2.0. Since the number of animals represented in the two means to be compared never falls below 41, values of "t" of 2.0 or greater than 2.0 may also be taken to indicate a statistically significant difference of means.

TABLE 1. EFFECT OF CERTAIN HORMONES ON PERCENTAGE ASH CONTENTS OF HUMERI OF NORMAL AND PARALYZED LIMBS IN THE RAT

Group	Number of animals	Treatment	Initial weight of animals mean ± S.E.[1] gm	Post-operative weight change of animals mean ± S.E. gm	Ash content of humeri of		Significance ratios[2]		
					Non-paralyzed limbs mean ± S.E. per cent	Paralyzed limbs mean ± S.E. per cent	I	II	III
A	31	Control	295.6 ± 8.59	11.7 ± 3.5	66.22 ± 0.422	65.53 ± 0.476			−1.08
B	20	Orchidectomy 3 days before operation to produce brachial paralyses	238.0 ± 7.96	19.1 ± 5.4	64.39 ± 0.558	62.77 ± 0.643	−2.61	−3.45	−4.48
C	19	Same as B plus 5 mg. testosterone propionate daily	218.4 ± 5.69	6.3 ± 3.7	63.21 ± 0.365	62.26 ± 0.263	−5.39	−6.01	−7.96
D	10	5 mg. testosterone propionate daily	299.0 ± 8.29	−20.0 ± 7.0	64.63 ± 0.449	64.17 ± 0.333	−2.01	−1.57	−2.65
E	19	50 micrograms estradiol dipropionate each alternate day	301.8 ± 8.13		66.31 ± 0.420	65.97 ± 0.345	+0.14	+0.74	−0.45
F	12	10 micrograms estradiol dipropionate each alternate day	293.5 ± 6.09	−33.1 ± 4.1	70.59 ± 0.206	70.25 ± 0.203	+6.02	+6.03	+5.79
G	21	5 micrograms estradiol dipropionate each alternate day	232.9 ± 2.37	− 5.9 ± 2.0	69.04 ± 0.252	68.16 ± 0.185	+5.73	+5.14	+4.21
H	14	Same as B plus 50 micrograms estradiol dipropionate each alternate day	283.0 ± 7.87		67.00 ± 0.278	66.79 ± 0.382	+1.18	+1.66	+0.83

[1] Standard error of the mean $= \frac{\text{S.D.}}{\sqrt{N-1}}$, where S.D. is the standard deviation of the mean and N the number of cases.

[2] Significance ratios (see text and text footnote 4). The Roman numerals at the head of the last three columns have the following meanings: I—Non-paralyzed humeri compared with non-paralyzed humeri of Group A; II—Paralyzed humeri compared with paralyzed humeri of Group A; III—Paralyzed humeri compared with non-paralyzed humeri of Group A. The plus and minus signs in the significance ratio columns indicate the direction of deviation of the mean tested from the mean result of Group A.

TABLE II. EFFECT OF CERTAIN HORMONES ON TOTAL DRY, FAT-FREE WEIGHT AND TOTAL ASH WEIGHT OF HUMERI OF NORMAL AND PARALYZED LIMBS OF THE RAT[1]

Group	Humeri of normal limbs				Humeri of paralyzed limbs				Degree of atrophy	
	Dry, fat-free weight		Ash weight		Dry, fat-free weight		Ash weight		Dry, fat-free weight[4] %	Ash weight[5] %
	Mean ± S.E.[2] mg./100 gms.	Sign. ratio[3]	Mean ± S.E. mg./100 gms.	Sign. ratio	Mean ± S.E. mg./100 gms.	Sign. ratio	Mean ± S.E. mg./100 gms.	Sign. ratio		
A	79.48 ± 0.932		52.64 ± 0.534		73.04 ± 0.865		47.80 ± 0.819		8.1	9.2
B	77.34 ± 1.45	−1.24	49.75 ± 0.886	− 2.79	66.10 ± 1.72	−3.60	41.47 ± 1.00	−4.90	16.8	21.2
C	83.92 ± 1.89	+2.11	53.02 ± 1.27	+ 0.28	76.44 ± 1.79	+1.71	47.64 ± 1.23	−0.11	3.8	9.5
D	75.79 ± 0.778	−2.16	49.01 ± 0.684	− 3.55	69.97 ± 1.24	−1.82	44.94 ± 0.990	−1.84	11.6	14.6
E	80.78 ± 1.72	+0.66	53.51 ± 1.15	+ 0.69	77.71 ± 1.88	+2.26	51.30 ± 1.23	+2.37	2.2	2.5
F	81.70 ± 1.16	+1.09	57.45 ± 0.770	+ 4.89	77.50 ± 1.13	+2.86	54.35 ± 1.25	+4.28	2.5	+3.2
G	88.68 ± 0.826	+7.39	61.25 ± 0.575	+10.97	81.79 ± 0.852	+7.21	55.76 ± 0.610	+6.40	+2.9	+5.9
H	80.91 ± 0.819	+0.95	54.20 ± 0.674	+ 1.70	78.46 ± 0.942	+3.76	52.41 ± 0.807	+3.44	1.3	0.4

[1] Mean dry, fat-free and mean ash weights per 100 grams of initial body weight of animals.
[2] Standard error of the mean (see footnote 1, Table I).
[3] Significance ratio (see text and text footnote 4). The plus and minus signs in the significance ratio columns indicate the direction of deviation of the mean tested from the mean result of Group A.
[4] Difference between the dry, fat-free weight of the humeri of paralyzed limbs and dry, fat-free weight of normal limbs of Group A (79.48) expressed as a percentage of the latter.
[5] Difference between the ash weight of humeri of paralyzed limbs and ash weight of humeri of normal limbs of Group A (52.64) expressed as a percentage of the latter.

the alteration of ash content was not statistically significant. The paradoxical result of both castration and plethora of an androgenic hormone producing a decrease in ash content of bones cannot be accounted for with complete assurance. It is possible that the massive doses of testosterone propionate which the members of Groups C and D received may have been so unphysiological as to have produced effects on the skeletal tissues which differ from those which would have resulted from smaller doses. The decrease in bone ash observed in Groups C and D was possibly not, wholly or in part, a direct effect of the androgen on the bones, but rather an indirect effect of an inadequate food intake by the animals receiving the testosterone propionate. It has been shown in other experiments (Armstrong, 1945) that deprivation of calcium or phosphorus or protein causes a decreased ash content of the humeri of normally innervated limbs, and an accentuated reduction of ash content of the humeri of paralyzed limbs. No observations as to food consumption were made in this study, but a comparison of the post-operative weight gains of the members of Groups C and D with that of the controls, as shown in Table I, supports the premise that the food intake of the treated animals was reduced. In this connection, it is also to be noted that the daily treatment of growing rats with 1 mg. of testosterone propionate has been reported to reduce the rate of growth in weight and length of normal (Rubinstein, Kurland and Goodwin, 1939) and castrated (Rubinstein, and Solomon, 1941) animals. The findings of these workers were explained as a result of an inhibition of the pituitary gland by the physiologically excessive quantity of androgen. Inhibition of growth hormone production probably occurred in the case of the members of Groups C and D. However, it appears unlikely that growth hormone deficiency had a significant relation to the results obtained with these animals since they were mature at the beginning of the experiments. It is known that rats continue to grow until an advanced age; nevertheless, the rate of growth of humeri of animals of the maturity of those employed in this study is so slow as to be negligible (Donaldson, 1915). Furthermore, the alteration of composition of the bones of Groups C and D is a reduction of mineral content and not an effect which would be expected to result from a reduction of growth.

As noted above, the castrated animals gained more weight than the members of the control group. This finding, which differs from that observed with animals castrated at weaning age (Rubinstein, Abarbanel and Kurland, 1939), is probably a result of lessened activity and increased fat deposition.

Effect of estradiol dipropionate on percentages of ash of humeri.— The humeri of the normal and paralyzed limbs of Groups F and G which received respectively 10 and 5 micrograms of estradiol dipropionate on alternate days were found to possess significantly higher

ash contents than the corresponding humeri of Group A. In point of fact, the humeri of the denervated limbs of these groups had higher contents of ash, on a percentage basis, than were probably present in their original preoperative state. This conclusion is indicated by comparing their ash content with that of the humeri of the normal limbs of Group A.

The administration of 50 micrograms of the same estrogen (Groups E and H) did not result in a significant alteration of the mean ash content of the humeri of either limb in comparison to those of the untreated control animals. The failure of large doses of an estrogen to produce an affect upon the composition of the skeleton which is caused by smaller doses of the same substance is probably a result of the largest dose of estradiol dipropionate markedly reducing food consumption and food utilization. This effect on food consumption and utilization was noted by Day and Follis (1941) to result from treatment of rats with estradiol benzoate. Evidence of reduced food intake by the animals receiving estradiol dipropionate in doses of 10 and 5 micrograms each alternate day was also obtained in this study from the finding that the animals of Groups F and G lost considerable weight during the experimental period (Table I). Unfortunately, the post-operative changes in weight of Groups E and H were not recorded, but it may be presumed that the effect of doses of 50 micrograms of the estrogen would influence body weight changes and food intake to degrees which would exceed that produced by an equal number of treatments with 10 micrograms of the same substance.

Effect of testosterone and estradiol propionates on the atrophy of humeri of the paralyzed limbs.—Since the composition of the humeri of the normally innervated limbs was affected by several of the forms of treatment, the degree of atrophy of the humerus of the paralyzed limb is not quantitatively described by differences in absolute weight or absolute ash weight between the two humeri of the same animal. A quantitative description of the atrophy of the humeri of the paralyzed limbs is presented in Table II in a manner which eliminates the influence upon the results of changes in the humeri of the normally innervated limbs. In this table the mean dry, fat-free and ash weights of the humeri per 100 grams of body weight on the day of operation are shown. The final body weights were not employed for this purpose since it is possible that the effect of the operations of body weight changes was not uniform in all animals. Furthermore, it was demonstrated, as mentioned above, that the post-operative body weight changes varied greatly among the several groups, presumably as a result of the various treatments. The use of the initial body weights as a means of relating the dry, fat-free and ash weights of the humeri of the animals in the several groups was further justified by the results of plotting the dry, fat-free and ash weights of the humeri of the normal limbs of Group A against the initial and also against the final

weights of these animals. In general, a direct relation of weight and of ash weight of the bones was noted with both the initial and final body weights and the "best" lines drawn through the points on the plots against initial and against final body weights were practically parallel. An indication of the degree of variation of weight and of ash weight of the humeri of the normal limbs of Group A in relation to the initial body weights of the animals is indicated in Table II by the standard error of the means.

The mean results and the significance ratios shown in Table II under the columns headed, "Humeri of Paralyzed Limbs" indicate (a) that orchidectomy increases the degree of bony disuse atrophy (Group B), (b) that treatment of orchidectomized animals with testosterone propionate in the dosage employed reduces the atrophy to a degree which does not differ from that obtained with uncastrated animals (Group C), (c) that administration of testosterone propionate to uncastrated animals in the dosage employed does not influence the bony atrophy to a statistically significant degree (Group D), and (d) that treatment of the animals with estradiol dipropionate reduces the atrophy of disuse occurring in the bones of the paralyzed limbs (Groups E, F, G and H). It will be noted that the effect of estradiol dipropionate in reducing skeletal disuse atrophy was found to be in inverse relation to the size of the doses of this estrogen, indicating that the smallest dosage was the nearest to the optimal in this regard and suggesting that an equal, or perhaps greater effect, might have been obtained from treatments using smaller amounts of estradiol dipropionate. In the same connection, it is also to be noted that a complete inhibition and reversal of the usual effect of paraylsis on the total ash content of the humeri of the paralyzed limbs was obtained in the animals receiving 10 and 5 micrograms respectively of estradiol dipropionate. This conclusion follows from the fact that the total ash contents of the humeri, per 100 grams of body weight, were found to be higher in the case of the humeri of the paralyzed limbs of Groups F and G than in the humeri of the normal limbs of the control group. This increase of ash weight of the paralyzed limbs is of statistical significance in the case of Group G, in which case also the dry, fat-free weight of the paralyzed limbs exceeds the corresponding weight of the humeri of the normal limbs of Group A.

The degrees of atrophy shown for the several groups in the last column of Table II were calculated on the assumption[5] that no hypertrophy or increase of total ash weight of the humeri of the normal limbs of Group A resulted from increased use of these limbs. These

[5] This assumption appears to be warranted because of the observation, made in connection with another study, that the dry, fat-free and total ash weights of humeri of unparalyzed limbs of rats with unilateral brachial paralysis of 21 days duration were not statistically significantly higher than those of the humeri of normal rats which had received the same dietary regimen.

calculated degrees of atrophy are an estimate, in percentage terms, of the probable alteration of the total dry, fat-free and total ash weights of the paralyzed limbs occurring during the period of the paralysis.

Effect of testosterone and estradiol propionates on total bone weight and total ash weight of humeri of normal limbs.—The mean results and significance ratios, shown in Table II, indicate the effects of the treatments on the humeri of the normal limbs. Except for Group C, the variations of total ash weight are in the same direction and in approximate proportion to the variations of percentages of ash of the same humeri shown in Table I. In the case of Group C, an increased total dry, fat-free weight of the normal humeri accounts for the fact that, in spite of a significantly lowered percentage of ash found in the humeri of the normal limbs, the total ash weight of these bones does not differ from that of the control group. The fact that opposite changes in the dry, fat-free and in ash weights were obtained with the humeri of the normal limbs of Groups C and D, even though both groups were treated with testosterone propionate in the same dosage, is possibly related to the circumstance that whereas the mean weight gain of the animals of Group C was 6.3 ± 3.7 grams, the post-operative weight change of the animals of Group D was -20.0 ± 7.0 grams. The lower initial weights of the members of Group C and the fact that these animals were castrated may account for their weight gain under conditions which produced a loss of weight by the animals of Group D. It is to be noted, as was the case with the humeri of the paralyzed limbs, that the greatest effect of estradiol dipropionate on the bones was obtained with the treatments of the smallest size.

Alteration of composition of the atrophic bones.—The humeri of the paralyzed limbs of Groups A, B, C and D, the groups in which significant degrees of bony atrophy occurred, were found to contain lower percentages of ash than was present in the humeri of the normal limbs of Group A (Table I). The atrophic humeri of the same groups also exhibited greater net losses of total mineral (ash) than of protein. This conclusion follows from the fact that in each of the above mentioned groups the degrees of atrophy calculated on the basis of ash weight are higher than those calculated on the dry, fat-free basis (Table II). These two results, the reduction of percentage of ash and of total ash, indicate that the composition of the bones which became atrophic was altered in the direction of that characteristic of osteomalacia rather than to that of osteoporosis.

The reduction of total ash weight of the humeri of the paralyzed limbs in each group is only partially accounted for by their lower ash content (percentage of ash). It was calculated, in the case of Group A, that 11.6 per cent of the lower total ash content of the bones of the paralyzed limbs is accounted for by their lower percentage of ash. The remainder of the reduction of total ash weight is, therefore, a

result of a lower amount of bone present in the humeri of the paralyzed limbs. The reduction in percentage of ash and in amount of bone of the atrophic humeri could have resulted either from an accentuation of the processes of bone resorption (and decalcification), from a reduced rate of bone formation (and calcification) or from a combination of these causes.

SUMMARY

Using mature male rats, the effects of orchidectomy and of the administration of testosterone and estradiol propionates on the humeri of normally innervated limbs and on the atrophy occurring in the humeri of paralyzed limbs have been investigated.

It was found that orchidectomy decreased the percentage of ash of both humeri as did also the daily administration of large doses of testosterone propionate. The percentages of ash of both humeri were found to be increased above their probable original ash content by the subcutaneous injection of 10 and of 5 micrograms of estradiol dipropionate on alternate days. This result was not obtained in animals receiving 50 micrograms of the same substance.

Orchidectomy was found to increase the degree of bony atrophy, an effect which was prevented by the administration of large doses of testosterone propionate. Treatment of the animals with estradiol dipropionate reduced the bony atrophy and the greatest effect was obtained with doses of 5 micrograms of the estrogen administered on alternate days, in which case the atrophy was prevented.

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THE ASSAY OF ANTIDIURETIC MATERIAL IN BLOOD AND URINE

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IN A STUDY of the nervous and hormonal control of urine formation, we have encountered the need of a method for detecting and, in some cases, estimating the amount of pituitrin in urine, blood and tissue extracts. Aqueous extracts of the neurohypophysis can be assayed by the method of Burn (1931), or one of its many modified forms, but the pituitrin content of blood and urine is so low that injection of the fresh material into rats is impracticable, and extraction of the hormone is a necessary preliminary to the assay. The antidiuretic material in urine can be isolated by dialysis or adsorbed onto a cellophane membrane and recovered by elution with acid (Gilman and Goodman, 1937; Martin, Herrlich and Fazekas, 1939; Walker, 1939; Ingram, Ladd and Benbow, 1939; Robinson and Farr, 1940; Ham and Landis, 1942; Frankel and Wakerlin, 1943). The antidiuretic substance in blood cannot be recovered by such a simple procedure (Levitt, 1936), but chemical extracts of blood may have an antidiuretic effect (Melville, 1937; Marx and Schneider, 1934). These extractions can be avoided, and fresh untreated blood from dogs or urine from almost any source can be injected intravenously, when dogs with experimental diabetes insipidus are used as assay animals (Hickey, Hare and Hare, 1941).

The method is sensitive enough to measure less than 0.5 milliunit (pressor) of pituitrin, even when it is contained in 50 cc. of blood or urine. An advantage of the method is that an antidiuresis produced by a vasomotor change with an attendant reduction in glomerular filtration can be distinguished from one resulting from an increased tubular reabsorption of water. When both factors are involved in an assay, as they sometimes are when a large volume of urine is injected, the rôle of each can be evaluated.

The basis of the assay is the change in the concentration of the urine, which is caused by a change in the tubular reabsorption of water. Since creatinine is excreted only by glomerular filtration in the dog, its concentration ratio has been used as an index of antidiuretic activity. Dogs with permanent polyurias, produced by sur-

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gical transection of the pituitary stalk, were used in preference to normal dogs with a sustained water diuresis (Melville, 1937), because the latter can release pituitrin in response to painful or emotional stimuli (O'Connor and Verney, 1942). Elimination of the endogenous source of pituitrin by degeneration of the pars nervosa of the pituitary has resulted in a larger percentage of successful assays, although it has not completely eliminated spontaneous antidiuresis. The intravenous route of administration avoids variations in absorption which may follow subcutaneous or intraperitoneal injection.

TECHNIQUE

All quantitative assays were performed in duplicate or triplicate, although the presence or absence of antidiuretic material may be detected by the use of only one assay. Female dogs with experimental diabetes insipidus were used in all assays. If the material assayed has a low creatinine content, as tissue extract or blood, the changes in urinary concentration of endogenous creatinine provide a satisfactory index of antidiuresis, even though the clearance of this chromogen material is not an accurate measure of glomerular filtration (Smith, Finkelstein and Smith, 1940). When urine is injected, however, the added creatinine produces a marked disturbance in the basal rate of excretion. This disturbance can be reduced to insignificance by giving the assay dog 3.0 gm. of creatinine in 100 cc. of water by stomach tube two hours before the beginning of the assay. In all cases, food was removed from the cage 24 hours, and drinking water 3 hours before the experiment, since food and water greatly exaggerate the variations in basal urine flow. During the assay, the dogs were kept without restraint on padded boards. Retention catheters, usually No. 16 Pezzar, were kept in the bladder throughout the experiment; urines were collected into volumetric flasks, and the bladder emptied at the end of each clearance period by washing with air. Bloods were collected at the midpoint of the first clearance period and at hourly intervals thereafter, but blood collections were omitted if the excretion of endogenous creatinine was the index of renal response. Creatinine was determined on tungstic acid filtrates of plasma and diluted aliquots of urine by the alkaline picrate method, and the concentration of creatinine in the urine was compared to that of the plasma at the midpoint of the clearance period, a value which may be obtained by interpolation on a concentration: time curve constructed from the analyses of the hourly blood samples. Since the responses to the doses of pituitrin measurable by this method last slightly less than forty minutes, two clearance periods were run each hour: a control period of twenty minutes and an assay period of forty minutes. The material to be assayed was administered intravenously. The response to a standard dose changes slowly throughout the assay, so that the response to an unknown amount can best be measured when it is preceded and followed by responses to the same standard dose; the shortest possible time for an assay is therefore three hours and twenty minutes.

CALCULATION

In earlier assays (Hickey, Hare and Hare, 1941), we collected urines at ten minute intervals and estimated the response to injections of pituitrin

from the area of the curve obtained on plotting the creatinine U/P against time. This method is most accurate when the basal creatinine U/P is constant, and in many assays, in which the basal U/P changed markedly, the results obtained by this calculation are grossly inaccurate. The percentile change in the creatinine U/P has proved a more generally applicable index

TABLE I. A DUPLICATE ASSAY OF 3.0 MILLIUNITS OF PITUITRIN

Procedure	Time	Urine flow	Creatinine		Clearance	U/P	Basal U/P	Δ U/P	% Δ U/P
			Plasma	Urine					
	min.	*cc./min.*	*mgm. %*	*mgm. %*	*cc./min.*				
Dog R									
Bladder emptied	0								
Blood 1	10.3								
1 mu	20.8								
Urine 1	21.3	2.72	9.8	172	48	17.6			
Urine 2	61.2	1.66	8.4	247	49	29.4	17.6	11.8	67
Blood 2	70								
3 mu	80								
Urine 3	81.3	2.63	7.6	135	48	17.7			
Urine 4	120.8	0.91	7.15	338	43	47.4	18.1	29.3	162
Blood 3	131								
1 mu	139.5								
Urine 5	141.1	2.26	6.7	124	42	18.5			
Urine 6	181.1	1.38	6.2	191	43	30.8	18.3	12.5	68
Blood 4	189.8								
Urine 7	200.8	2.65	5.6	102	48	18.1			
Dog Q									
Bladder emptied	0								
Blood 1	9								
1 mu	18.7								
Urine 1	19.8	2.35	15.1	269	42	17.8			
Urine 2	57.8	1.6	14.2	382	43	26.9	20	6.9	34
Blood 2	67								
3 mu	76								
Urine 3	77.2	1.83	13.4	298	40	22.3			
Urine 4	116.8	0.90	12.5	551	40	44	22.3	21.7	97
Blood 3	126								
1 mu	135.6								
Urine 5	136.7	1.85	11.6	258	41	22.3			
Urine 6	176.5	1.53	10.8	289	41	26.8	20.4	6.4	31
Blood 4	185								
Urine 7	195.1	2.24	10.3	192	42	18.6			

of antidiuresis. The basal creatinine U/P for the assay period is the average of those for the control periods immediately preceding and following.

When the excretion of endogenous creatinine was measured during the course of an assay, the percentile change in the creatinine concentration of the urine was the indicator of antidiuretic potency. This value is really identical with percentile change in creatinine U/P, as the plasma concentration of endogenous creatinine does not change to a measurable extent during an assay, and P is therefore a constant which may be ignored.

XUM

When duplicate assays agreed within 0.3 mu, the average was considered an acceptable value, but when duplicates differed to a greater extent, the assay was repeated. If two of the triplicate assays agree within 0.3 mu and the third was erratic, the average of the two was accepted.

RESULTS

Assay of Pituitrin. Twelve duplicate assays of pitressin, in which doses of from 0.1 to 5.0 mu were compared to standard doses of 1.0

TABLE II. DUPLICATE ASSAY OF 1.0 MILLIUNIT OF PITUITRIN

	Time	Urine flow	Urine Creatinine	Basal Urine Creatinine	Δ Urine Creatinine	% Δ Urine Concentration
	min.	*cc./min.*	*mgm. %*	*mgm. %*	*mgm. %*	
Dog R						
Bladder emptied	0					
1 mu	19					
Urine 1	20.3	1.73	20.7			
Urine 2	60	0.87	40.7	22.3	18.4	83
1 mu	80.4					
Urine 3	81.3	1.47	23.9			
Urine 4	119.8	0.63	50.6	25.3	25.3	100
1 mu	138.7					
Urine 5	140.1	1.29	26.7			
Urine 6	180.7	0.69	46.4	27.9	18.5	66
Urine 7	199.4	1.10	29.1			
Dog Q						
Bladder emptied	0					
1 mu	18.6					
Urine 1	20.0	5.38	10.0			
Urine 2	60.0	3.79	12.9	10.7	2.2	26
1 mu	79.0					
Urine 3	80.2	4.32	11.5			
Urine 4	120.0	3.09	15.9	12.2	3.7	30
1 mu	138.0					
Urine 5	140.0	3.79	13.0			
Urine 6	179.8	3.03	15.9	10.8	4.1	38
Urine 7	199.1	4.78	8.5			

mu, established the useful range of dosage from 0.3 to 3.0 mu. Doses of 0.1 mu could be detected, but were estimated at 0.05 to 0.2, an error of 100%. Doses of 3.0, 4.0 and 5.0 mu never assayed more than 3.0, which is therefore the upper limit of the dosage range. Any assay of an unknown giving a value larger than 2.0 mu is open to the suspicion that the dose was supramaximal, and the assay should be repeated with a smaller quantity of the material.

Assays with endogenous and exogenous creatinine clearances are compared in Table I and II. Table I shows a duplicate assay of 3.0 mu with exogenous, and Table II a duplicate assay of 1.0 mu with endogenous creatinine.

The results of the three hour assays of known doses of pituitrin are summarized in Figure 1, in which the dose is plotted against the amount estimated from the response.

In a large portion of our experiments it was desirable to make comparisons with standard pituitrin solutions of the antidiuretic potency of bloods or urines collected before and after various procedures on the donor dog. This required prolonging the assays for

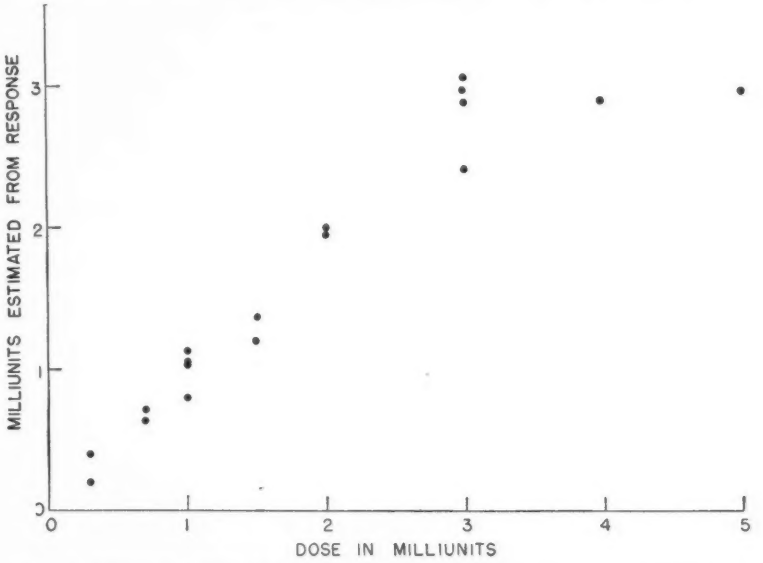

FIG. 1. Assays of known doses of pituitrin. The amount injected is plotted on the abscissa; the amount estimated from the response on the ordinate. Of twelve duplicate assays performed, only eight are included; the duplicates failed to agree in the other four. Single assays of 4.0 and 5.0 mu are included to show that doses in this range are supramaximal.

one or, in a few instances, for two hours. While this almost certainly diminished the accuracy of the results, it permitted acceptable evaluation of the amount of pituitrin in the fluids injected. One of these prolonged assays, done in duplicate is shown in Figure 2.

Assay of the antidiuretic content of blood. The blood was drawn into an oiled syringe without an anticoagulant, since heparin may be an antidiuretic (Walker, 1938), and quickly injected into the assay dog through a 16 gauge needle previously introduced into the external jugular vein. Only one reaction has ever occurred in the recipient,[1]

[1] This reaction, consisting of severe intravascular hemolysis, hemoglobinuria, chills and fever, occurred in a dog that had received a total of 300 cc. of blood by several transfusions from this same donor. There was a six week interval between the last of these transfusions and the one which caused the reaction.

but a transfer of more than 50 cc. has not been attempted. Of twenty-four assays of blood from normal dogs, one has given a positive response. Since Heller (1935) has shown a rapid inactivation of pituitrin by blood in vitro, it was suspected that our transfer time, short

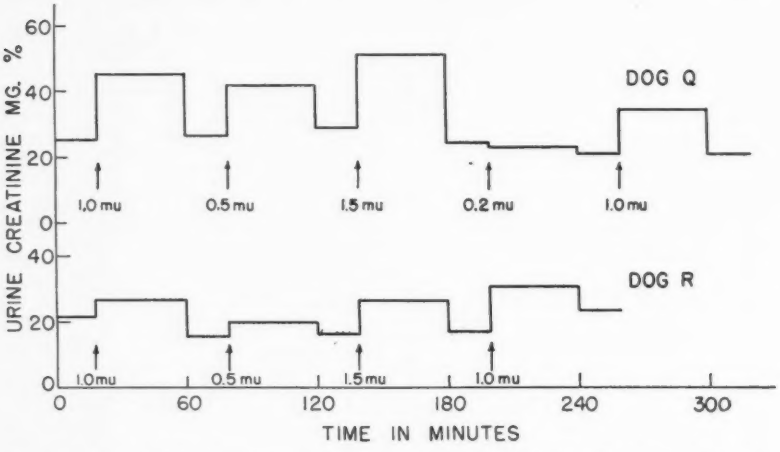

FIG. 2. A four and five hour assay for known doses of pituitrin. The dose of pituitrin injected at the time indicated by each arrow is expressed in milliunits.

though it was, was sufficient for the circulating pituitrin to be destroyed. This was shown not to be the case in the following experiment: 50 cc. of blood from a dog with diabetes insipidus was injected into an assay dog; an hour later a second aliquot of blood from the

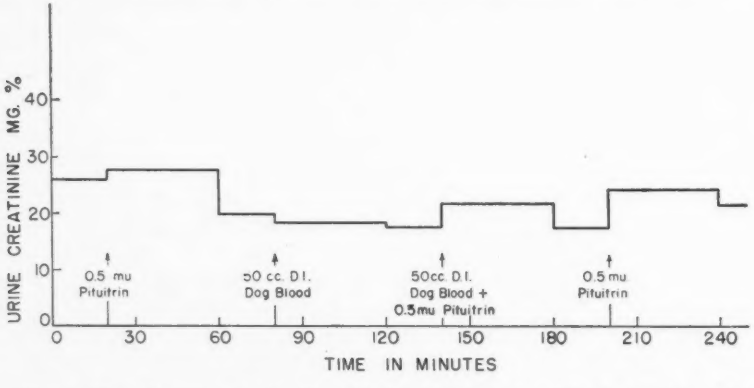

FIG. 3. The estimation of pitruitin added to blood *in vitro*. The first and fourth injections are of pituitrin in 5.0 cc. of 0.9% NaCl solution. The second injection is of 50 cc. of blood from a dog with diabetes insipidus and presumably without any circulating pituitrin; the third is of 50 cc. of blood from the same donor but with 0.5 mu of added pituitrin.

same donor was drawn into a syringe containing 0.5 mu of pituitrin, mixed and injected into the same recipient. The first blood caused on change in the recipient's urine flow; the second produced about the same antidiuresis produced by 0.5 mu of pituitrin alone (Fig. 3).

Assay of Urine. We have previously shown that fresh urine may be used for qualitative assays, when diabetes insipidus dogs are used as test animals. The present experiments, which are much more extensive, have shown that such assays may even provide an estimate of potency. The most satisfactory evidence that the urinary assays were correct was obtained by adding pituitrin to aliquots of urine collected from a dog with diabetes insipidus. The untreated urine produced a slight diuresis in the assay dog, but the urine plus pituitrin produced an antidiuresis commensurate with the amount of added pituitrin (Fig. 4).

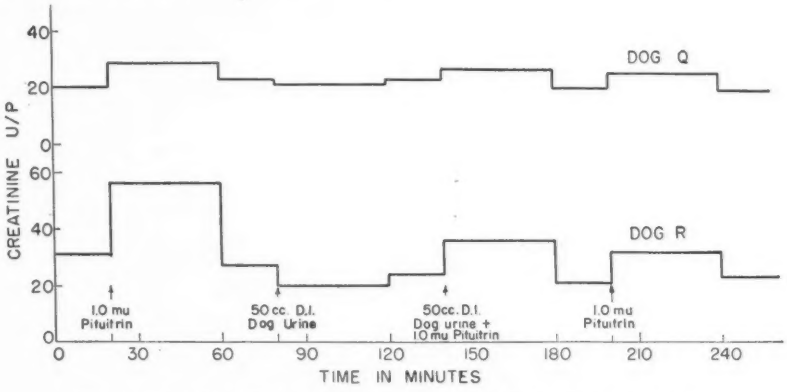

FIG. 4. Duplicate assay of pituitrin added *in vitro* to urine from a dog wish diabetes insipidus.

While the species specificity which limited the blood assays to dog's blood does not, of course, hold for urine, several precautions are necessary. Urine from patients or dogs with urinary tract infections cause prolonged oliguria or even anuria, which preclude evaluation of the assay. Only freshly collected specimens should be used, and even when fresh clear urines are used, the dog receiving the injection may retch and pale. These reactions, even though they obviously include a marked vasomotor change, do not, as a rule affect glomerular filtration or the tubular reabsorption of water. Accurate assays, both negative and positive, have been obtained under these circumstances.

An occasional urine contains a powerful diuretic, which becomes effective about an hour after injection. This delayed effect seriously distorts, or even prohibits evaluation of the response to subsequent injections. In five assays the diuresis was not modified by the injection of 1.0 to 2.0 mu of pituitrin. The nature of the diuretic is not

known, but the volume and osmotic pressure of the urine injected have been excluded.

DISCUSSION

Most of the methods for the bioassay of pituitrin depend upon its effect on blood pressure or smooth muscle. Those methods by which the antidiuretic activity is measured require a relatively pure and concentrated solution of the hormone, especially those which use mice (Jeffers et al., 1942) or rats (Burn, 1931) as subjects. When the anesthetized rabbit is used according to Walker's (1939) technique, it is possible to use larger volumes of more dilute solutions of the hormone. When a dog of 12 to 20 kg. is used, the volume of injection may be varied from 0.1 to 100 cc. without appreciably altering the accuracy of the assay. This tolerance of large intravenous injections is fortunately attended by a high degree of sensitivity. 0.1 mu is often sufficient to produce a detectable change in urine concentration, and 0.5 mu is well above threshold. Unfortunately, the response of the same dog to the same dose of pituitrin is not constant from day to day; in fact, it varies so from hour to hour that some assays must be discarded. The factors which cause these fluctuations in sensitivity and in basal rate of urine flow are not known, but they occur much less frequently when the animals are on a constant diet and are fasted twenty-four hours before the clearance.

The effect of pituitrin as measured by changes in urine concentration is inversely related to urine flow in dogs wish diabetes insipidus. When the urine flow of the assay dog is less than 1.5 cc./min., the maximal dose of pituitrin is less than 2.0 mu, and decreases as the rate of urine flow falls. This led at first to serious errors in comparisons of the effects of known doses of standard pituitrin solution, but it soon became apparent that all the doses were supramaximal. It is essential therefore, that the basal urine flow be at least 1.5 cc./min., and one of 2 to 5 cc./min. is desirable. The first assay in Table II was included to illustrate this dependence of accuracy upon a minimal rate of urine flow. In this case is found the largest error in the four experiments reported in detail. However, this increased sensitivity at low rates of urine flow, may be used to advantage in assaying material of extremely low potency, such as blood, but in such assays the reference dose of pituitrin should be reduced to 0.5 mu or even less (Fig. 3).

SUMMARY

Antidiuretic material in tissues, blood and urine may be estimated when a dog with experimental diabetes insipidus is the test animal, and creatinine U/P ratios are followed after the intravenous administration of the unknown material. Large volumes of untreated urine or dog's blood may be injected. The response to the unknown is compared to the responses to two standard doses of pituitrin, one preced-

ing and one following. Duplicate assays should agree to within 0.3 mu to be acceptable. The useful range of dosage is 0.3 to 3.0 mu of pituitrin, when the basal urine flow is more than 1.5; at lower urine flows the threshold and maximal doses are lower.

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XUM

ANTIDIURETIC MATERIAL IN THE SUPRAOPTIC NUCLEUS

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TWO FUNCTIONS have been attributed to the supraoptic nuclei of the hypothalamus: trophic innervation of the neurohypophysis and secretion. The first is well established, and according to Magoun and Ranson (1939) "few connections in the nervous system rest upon the volume of supporting evidence which has been brought forward for that between the supraoptic nuclei and the neurohypophysis." In silver stained sections of normal tissue, it is possible to follow the origin and course of the nerve fibers which pass from the hypothalamus down the pituitary stalk to the posterior lobe (Fig. 1). After transection of the pituitary stalk, not only is there a degeneration of the fibers distal to the lesion and an atrophy of the posterior lobe, but a retrograde process leads to the death and disappearance of 80 to 95% of the neurons in the supraoptic nuclei (Rasmussen, 1940; Heinbecker and White, 1941; Magoun and Ranson, 1939). Outside of its trophic influence on the pituicytes, the function of this tract is much less clear than its anatomy. Reflexes, originating in peripheral nerve stimulation and leading to the release of pituitrin, have been described (Rydin and Verney, 1937; Chang et al., 1939; Haterius, 1940). In addition it has been suggested that the supraoptic neurons, since they have an extremely generous blood supply, might serve as chemoreceptors which, in response to changes in plasma composition, could regulate the release of pituitrin and thereby the renal excretion of water (Craigie, 1940; Ingram, Ladd and Benbow, 1939; Hare, Hare and Phillips, 1943).

The secretory activity of supraoptic neurons is based solely on cytological evidence. The Scharrers (1945), in a recent review, have arrayed this evidence of secretory neurons in the hypothalamus which, in the mammal, are localized in the supraoptic and paraventricular nuclei. On the basis of cytological studies, Gaupp and Scharrer (1935) have postulated the production by these neurons of an antidiuretic substance and its participation in the regulation of water exchange.

If the Scharrers' hypothesis is correct, it should be possible to demonstrate an antidiuretic substance in the supraoptic nuclei, and this we have done. Our evidence cannot indicate more than the pres-

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ence of antidiuretic substance; its site and mechanism of formation must remain hypothetical. The assay of aqueous extracts of the hypothalamus has shown a high concentration of antidiuretic material in the region of the supraoptic nuclei of dogs. When the nuclei were degenerated as the result of hypophysectomy or of cutting the pituitary stalk, the antidiuretic potency of the hypothalamus was greatly diminished.

MATERIALS AND METHODS

In one series of experiments the antidiuretic potency of extracts of the neurophypophysis and hypothalamus of normal dogs was compared to

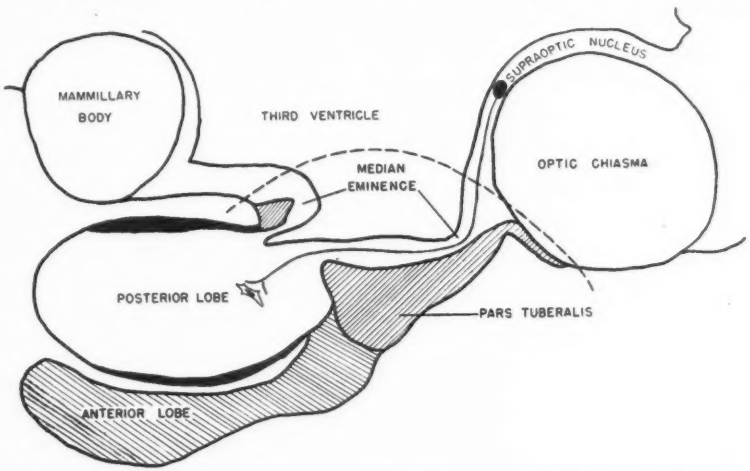

FIG. 1. Schematic drawing from a sagittal section through the hypothalamus and pituitary of the dog. The broken curve represents the line along which the tuber cinereum and pituitary were cut away from the hypothalamus. The supraoptic nucleus, which actually lies lateral to this plane of section, is projected onto the drawing to show the course of the nerve fibers which end about pituicytes in the pars nervosa.

standard pituitrin solutions. In a few cases the hypothalamic tissue was divided and a small block containing the supraoptic nuclei was cut out and extracted separately. In a second series, the pituitary of the donor dogs had been excised or detached from the hypothalamus two weeks to four years before the animal was killed. The whole hypothalamus was extracted in the majority of these cases. In three, however, the supraoptic regions were isolated and extracted as described above, and compared in antidiuretic potency to similarly prepared extracts of normal supraoptic nuclei.

Fifteen dogs were used in the experiment. Seven were normal, two were hypophysectomized, and in six the pituitary stalk had been cut. Of the last six, three had a polyuria of 4 to 6 liters per day of more than two years duration.

About two hours before their tissues were to be assayed, the dogs were killed by decapitation under barbiturate anesthesia. The floor of the skull was removed to expose widely the region of the optic chiasm and the pitui-

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tary. The dura was then opened, and the pituitary, or the stump left after hypophysectomy, was severed from the hypothalamus at a level which assured that no remnant of the neurohypophysis was left attached (Fig. 1). This was accomplished by removing with small scissors the caudal part of the optic chiasm, the floor of the third ventricle and part of its lateral walls. The hypothalamic block of tissue then removed extended from 2 mm. rostral to the optic chiasm caudally to the interpeduncular fossa, laterally to the lateral geniculate bodies, and dorsally to a plane passing through the anterior commissure and the aqueduct of Sylvius. The neurophypophysial tissue, assayed in four cases, included the tuber cinereum, the pituitary stalk and the pars neuralis. The blocks containing the supraoptic nuclei weighed 100 to 200 mg., the neurohypophysis 20 mg., and the hypothalamus 2 to 3 gm. The tissues, collected in tared bottles, were weighed and then ground with sand in 0.25% acetic acid. After thorough maceration, the suspension was transferred to a flask and made up to 1.0 liter with 0.9% NaCl. Further dilutions, when necessary, were made from this stock which was kept at 2° C. For controls, similar extracts were made of cerebral cortex.

The antidiuretic potency of these extracts was estimated by the procedure described in the preceding paper (Hare et al., 1945).

RESULTS

A duplicate assay of the hypothalamus of a 6.6 kg. normal male dog is shown in Figure 2. The antidiuretic content of this hypothalamus

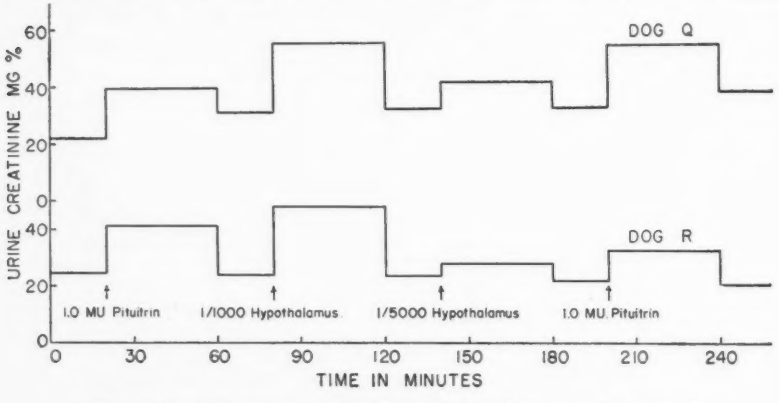

FIG. 2. Duplicate assay comparing the hypothalamus of a normal dog to a standard pituitrin solution.

was estimated as the equivalent of 1.5 to 2.0 units of pituitrin. In other normal dogs the estimates have been higher, 3.5 to 5.0 units, but the dogs weighed 9 to 14 kg.

Assays of other nervous tissues for pituitrin have been consistently negative (Sato, 1928; Abel, 1924; Hickey, Hare and Hare, 1941), but our present findings on the hypothalamus of the dog led us to assay again similar extracts of the dog's cerebral cortex. Figure 3 shows a negative assay of this material.

The next experiments were attempts to localize further, within the hypothalamus, the origin of the antidiuretic material. The following protocol is representative.

Experiment, Dec. 15, 1944. A 7.0 kg. female mongrel was killed and the base of the skull rapidly removed. The pituitary, its stalk and the tuber cinereum were cut off and discarded. The optic chiasm, the optic tracts and the contiguous diencephalic tissue were removed in one piece which weighed 185 mg. The rest of the hypothalamus weighed 6.726 gm. Extracts of the two tissues were assayed in duplicate (Fig. 4). The supraoptic block contained the equivalent of 1200

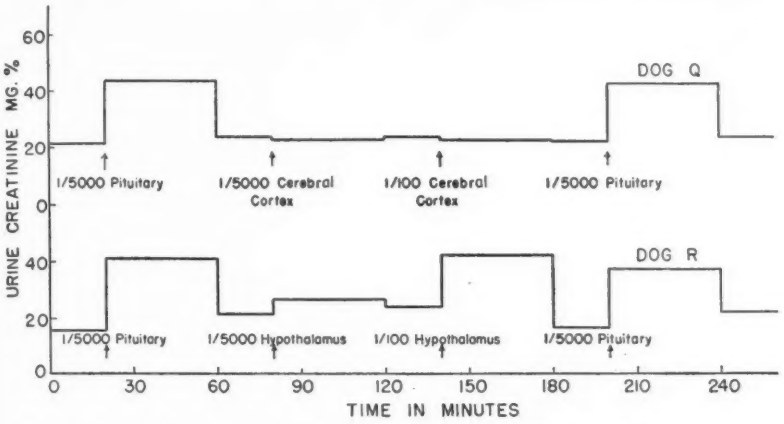

FIG. 3. Comparison of extracts of cerebral cortex and of the hypothalamus of a normal dog to an extract of its neurohypophysis. The pituitary weighed 20 mg. so the amount contained in 1/5000 of the extract was equivalent to 4.0 μ gm. of fresh tissue; the block of cerebral cortex weighed 2.6 gm. and 1/100 of the extract is equivalent to 26,000 μ gm. of fresh tissue; the hypothalamus weighed 2.0 gm. and 1/5000 of the extract was equivalent to 400 μ gm. fresh tissue. The injection of 1/1000 of the hypothalamus provided a supramaximal dose.

to 1400 mu. of pituitrin; the rest of the hypothalamus, which weighed more than 30 times as much, contained less than 300 mu.

In making this comparison on a weight basis of the antidiuretic content of the two blocks of tissue, it should be kept in mind that the optic tracts and chiasm constituted the great majority of the supraoptic specimen. In other experiments, the optic paths were dissected from the hypothalamus and a small block, intended to include the supraoptic nucleus, was excised from each side of the hypothalamus. These specimens from the supraoptic region weighed 106 mg. and assayed 4 units of pituitrin; the rest of the hypothalamus exclusive of the optic chiasm and tracts weighed 2.735 gm. and assayed 1.0 unit. The antidiuretic content of the supraoptic nuclei is, on a weight basis, more than a hundred times that in the rest of the hypothalamus.

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In the fresh tissue, the exact site and limits of the supraoptic nuclei cannot be identified with sufficient clarity to permit a dissection, in the gross, which would assure the inclusion of all the cells in the tissue removed. We, therefore, resorted to the selective degeneration of the cells in these nuclei by cutting their processes passing down the pituitary stalk. An assay of the supraoptic region and of the remainder of the hypothalamus from one of these dogs is shown in a Figure 5. This dog, a 12.5 kg. male, was killed 16 days after hy-

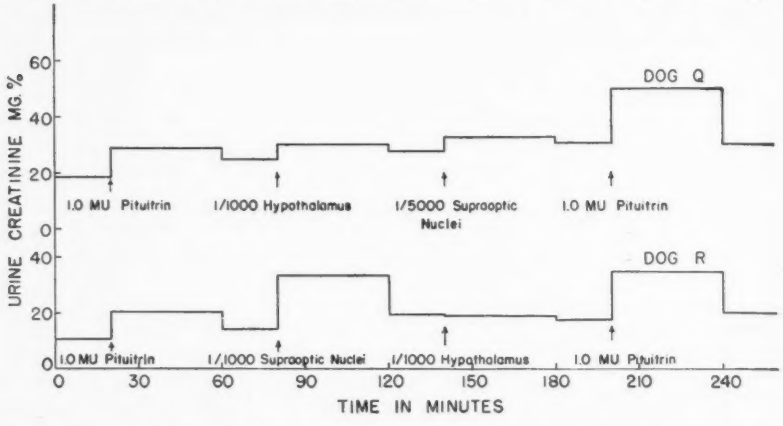


FIG. 4. Assay comparing the potency of extracts of the supraoptic region and of the rest of the hypothalamus with a standard pituitrin solution.

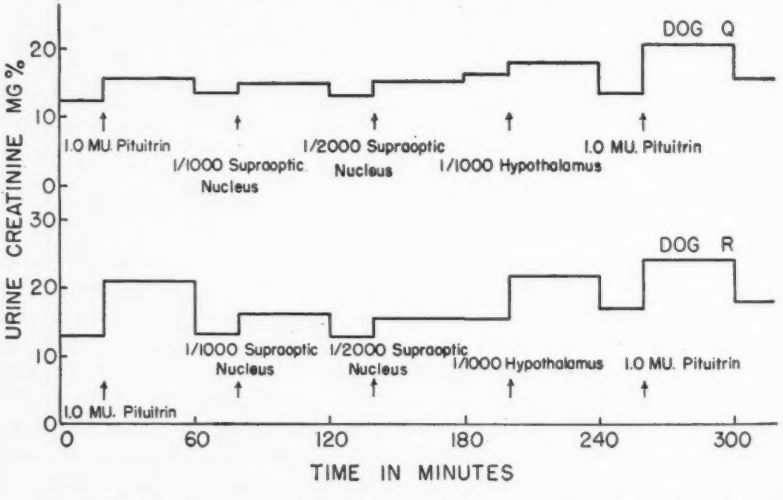
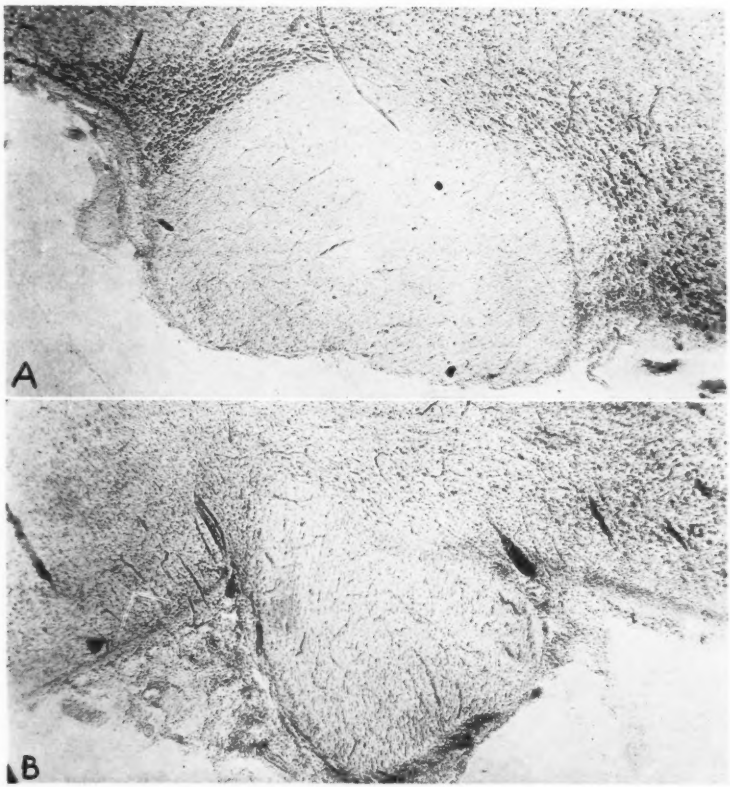


FIG. 5. Duplicate assay comparing extracts of the supraoptic region and of the rest of the hypothalamus of a dog 16 days after hypophysectomy with a standard pituitrin solution. Compare with fig. 4.



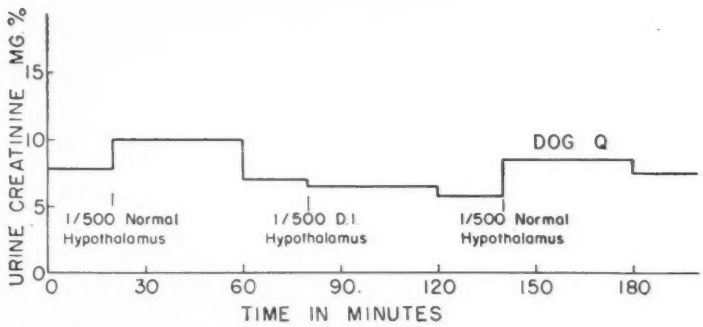


FIG. 6. Comparison of the histological appearance and hormonal content of the supraoptic region of a normal and of a polyuric dog. The photomicrographs of 50 μ sections stained with cresyl violet represent the sections showing the largest number of neurons in the supraoptic nucleus. Normal dog (a), polyuric dog (b). The opposite halves of the hypothalamus were trimmed until only the supraoptic region remained. The assay shows that the normal contained the greater amount of antidiuretic material.

pophysectomy and 2 days after the onset of a polyuria. The supraoptic tissue contained the equivalent of 400 to 500 mu. of pituitrin; the rest of the hypothalamus 700 to 800 mu. This is a reduction of about 75% in the potency of the supraoptic tissue. Much more severe loss of potency was found in one of the dogs with a polyuria of 3 years duration resulting from transection of the pituitary stalk. The whole hypothalamus, exclusive of the tuber cinereum, assayed only 100 mu.

The most satisfactory correlation between the neuronal atrophy in the supraoptic nuclei and the loss of antidiuretic potency was obtained by comparing both factors in a normal and chronically polyuric dog of the same sex and size. This was accomplished by dividing the hypothalamus of each along the midline; one-half was fixed in formalin for histological preparation, the other extracted for assay. The results are shown in Figure 6.

DISCUSSION

The presence of significant amounts of antidiuretic material in the hypothalamus, first reported by Abel (1924), has been confirmed. A few years later, Sato (1928) found that the hypothalamus of the normal dog contained an oxytocic and pressor substance which increased in amount following hypophysectomy. These findings, apparently directly contrary to ours, have an acceptable explanation in the anatomical intricacies of the hypothalamic-pituitary complex. Sato prepared extracts of the tuber cinereum, the floor of the third ventricle which forms a funnel leading into the pituitary stalk. His tissue very probably contained, therefore, the median eminence which, like the posterior lobe, contains pituicytes. Blount (1941) has shown that these cells produce vasopressin, and according to Gersh and Brooks (1941) they hypertrophy after hypophysectomy. Since we excised and discarded the tuber cinereum, there is no real contradiction between our observations and Sato's findings.

Although we found a large reduction in the amount of antidiuretic material in the hypothalamus of dogs with severe and long lasting polyuria, a surprisingly large quantity of the material was present: in no case was there less than 100 mu. Furthermore the posterior lobe and the tuber cinereum contained several hundred milliunits. In a normal dog the pars nervosa, weighing about 20 mg. contains about 15 units of pituitrin; the supraoptic region, weighing about 100 mg., contains 1.5 to 5.0 units; and the rest of the hypothalamus, weighing 2 to 4 gm., contains less than 1.0 unit. A residual supply of 300 to 700 mu. in the polyuric dog seems inconsequential when compared to the store of approximately 20 units available to the normal dog. This remaining source of pituitrin may exert some influence, since only 5 to 10 mu. per hour is necessary to maintain a normal urine flow (Shannon, 1942). Even the most severe diuresis yet seen in experimental

diabetes insipidus may not represent the maximal which would obtain in the complete absence of pituitrin.

The presence of antidiuretic material in the atrophic hypothalamic-hypophysial tissue of polyuric dogs may provide an explanation for the slow variations in urine flow, the occasional sudden antidiuresis, or the diminished urine flow during dehydration of dogs with experimental diabetes insipidus. Of greater significance is the fact that the supraoptic neurons contain 15 to 25% as much antidiuretic material as the neurohypophysis. This is foreign to the concept that these neurons serve only as the final common pathway in a reflex system regulating the production and release of pituitrin by the pituicytes. Our present evidence cannot justify the hypothesis of the Scharrers that pituitrin may be secreted by the supraoptic neurons and transported along their axis cylinders to the pars nervosa. Some broader concept of supraoptico-hypophysial relations is indicated and much more information must be collected before a new hypothesis can be advanced.

SUMMARY

The supraoptic region of the dog's hypothalamus contains an amount of water soluble antidiuretic substance equivalent to 1.5 to 5.0 units of pituitrin. Degeneration of the supraoptic nuclei, resulting from hypophysectomy or surgical transection of the pituitary stalk, is attended by a severe loss of this antidiuretic material. This has been determined from assay of the hypothalamus or supraoptic region of 15 dogs. Seven were normal, two were hypophysectomized, and six had the pituitary stalk severed.

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XUM

THE EFFECT OF ADRENALECTOMY ON THE EPIPHYSEAL CARTILAGE OF THE RAT

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THE BEHAVIOR of the proximal epiphyseal cartilage of the rat's tibia is an excellent criterion for studies of the relation of internal secretions and nutrition to growth or to each other. It is an extremely active point of growth in the young rat (Dodds and Cameron, 1934; Ingalls, 1941) and does not disappear until late in life when union of the epiphysis with the shaft takes place after a thousand days of age (Dawson, 1925). At present it is being considered extensively as an important locus of action of the pituitary growth hormone, which has been called a "chondrotrophic hormone" (Freud, Levie and Kroon, 1939; Ray, Evans and Becks, 1941).

In 1941 Ingalls and Hayes demonstrated a stunting of the epiphyseal cartilage and a failure of endochondral bone formation in eleven rats from one to two weeks after adrenalectomy, and suggested that atrophy of the adrenal cortex might be a major factor in the interruption of growth consequent to hypophysectomy. The lability of the epiphyseal cartilage under changing nutritional states (Harris, 1933; Becks, Simpson, Li and Evans, 1944) makes it imperative to consider inanition, at least as a contributing factor, in growth changes in adrenocortical insufficiency. Groat (1941) has shown that in the adrenalectomized rat a loss of appetite (which may be restored by the administration of salt) leads to a self imposed starvation which in itself might be sufficient to cause death. The symptoms following adrenalectomy, therefore, result from simultaneous adrenocortical insufficiency and nutriment deficiency. Mulinos and Pomerantz (1940) have likewise pointed out the importance of inanition in the sequelae of hypophysectomy and have actually called the results of food restriction "pseudohypophysectomy."

In 1941 we presented evidence that castration of the male rat 'releases' corticotropin from the pituitary gland. If such an extension of the 'end organ' hormone hypothesis is generally valid we might expect that removal of any endocrine gland which is stimulated by a hormone from the anterior pituitary would 'release' all the tropins

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[1] We are indebted to David Silver, Frank A. East and Frederick Sargent II for assistance with this problem.

of the pituitary as well as the one specific for the organ removed. Adrenalectomy, therefore, should be followed by a 'release' of growth hormone which could be detected by a *widening* of the epiphyseal cartilage. This paper presents further evidence for this view.

METHODS

Rats of the Long-Evans hooded strain were used. The majority were males, and the few females employed are so designated in the descriptions of results. No significant sex differences in behavior of the epiphyseal cartilage were revealed. For all procedures about half of each litter, or one of a pair of litter mates, were used as experimental animals and the others were kept as controls. These latter were blank operated if the experimental group were adrenalectomized; and given salt solution (0.5 per cent sodium chloride, 0.5 per cent sodium citrate) in place of drinking water when it was used for the adrenalectomized rats. Age in days when given is that at the time of measurement of the cartilages. For measurement, by means of a micrometer ocular the quick method of staining the split tibia with silver nitrate recommended by Evans, Simpson, Marx and Kibrick (1943) was employed.

RESULTS

Adrenalectomy. From 5 to 11 days after adrenalectomy the proximal epiphyseal cartilages of the tibias of 15 out of 19 young growing rats were definitely narrower than the average width of those of their litter mate controls (Fig. 1, A; Table 1). In 13 the difference was between 20 and 38 per cent, and in two it was 42 and 79 per cent. Of the other four one showed no change while the cartilages of three were less than 20 per cent narrower than the controls (15–19%), which is probably not significant. These findings confirmed those of Ingalls and Hayes (1941). Such narrowing of the tibial cartilage was not seen during the first post-operative week in 8 fully grown adrenalectomized rats (181, 188, and 244 days old).

Adrenocortical transplantation. The widths of the epiphyseal cartilages of 12 adrenalectomized rats with autoplastic dorsal intramuscular transplants of adrenal cortex 60 to 77 days after operation were the same as those of their blank operated litter mates (Table 1). In such animals the appetite, general activity, and nutritional state are like those of normal rats, although they are more susceptible to stress of various sorts.

Food consumption and body weight. Daily food consumption of normal rats was calculated from data on 65 normal males and 13 females which were followed for periods up to 6 weeks. In the males food intake increased during the first seven weeks of life from 13–16 gm. (av. 14) at 30–40 days of age, and 15–19 gm. (av. 17) at 40–50 days, to an adult consumption of 16–20 gm. daily (av. 18) at 50 to 250 or more days of age. Both "Purina Dog Chow" and "Rockland Rat Diet" were tested, but no difference was detected. Female consumption was less, increasing from about 11 gm. daily at 40–50 days to 12–15 gm. (av. 13) for adults 50–80 days old.

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Seventeen male and five female rats (of 12 litters), 54–69 days old, were adrenalectomized and the daily food intake was followed for 6 or 7 days. Then salt solution was substituted for their drinking water and food intake was measured for 7 or 8 days more. The food intake dropped after adrenalectomy and no average figure of much significance can be given because of the variability, but it was roughly half that before operation (av. 55%). Body weight loss for the week was

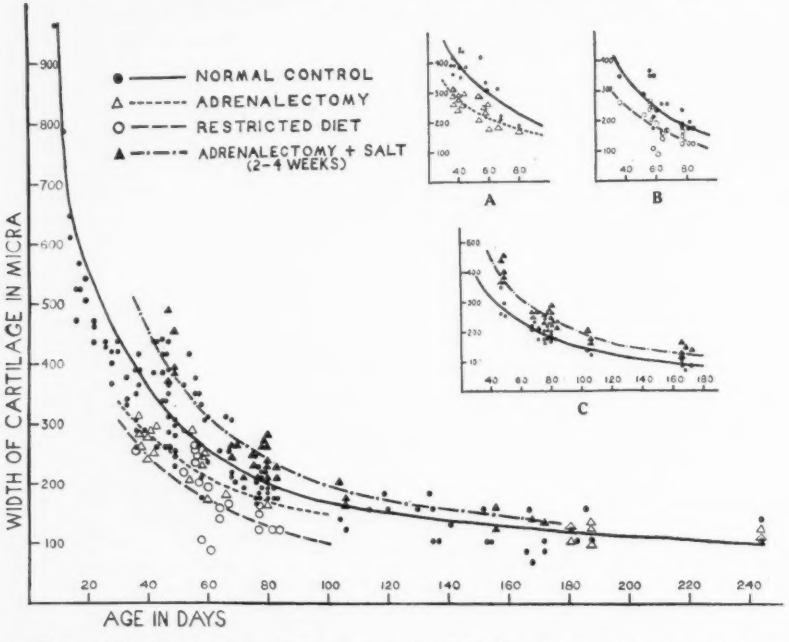

FIG. 1. The effect of (A) adrenalectomy, (B) restricted diet, and (C) adrenalectomy plus salt treatment on the width of the proximal epiphyseal cartilage of the rat's tibia. When the data were plotted on double logarithmic paper straight line graphs were obtained, and the theoretical curves were calculated according to the method of averages to fit the formula $y = ax^b$.

from 1 to 29 grams. Upon supplying salt water the rats' appetites increased (see Groat, 1941) although food intake did not return to the normal level, but only to about three-fourths of it (av. 72%). The rats gained weight during the week in amounts about equal to their previous loss plus one week's normal growth, which left them about one week behind their controls. The food consumption and body weight increase of 12 male and 6 female normal litter mates of these rats, which were subjected to the same shift from drinking water to saline solution, showed no significant deviations from the normal for their age.

Restricted diet. In order to imitate the nutritive conditions in adrenalectomized rats the daily rations of 19 normal rats (10 males, 9 females) were restricted to about half normal consumption, i.e. about 8 gm. for young growing rats and 10 gm. for adults. Their litter mate controls (9 males, 8 females) were allowed food *ad libitum*. At the end of a week the rats on restricted diet had lost weight in varying amounts (0 to 47 gm.) and the controls had gained (3 to 33 gm.). The changes were not unlike those exhibited during the first

TABLE 1

Experimental procedure	Number of litters	Number of rats		Age at end of experiment (*days*)	Duration of experiment: from operation (*days*)	Width of proximal epiphyseal cartilage of tibia (*average & range, micra*)		
		Controls	Experimental			Controls	Experimental	Per cent difference
Adrenalectomy	6	13	10	36–43	5–11	403 (350–446)	285 (245–312)	−29 (−20 to −79)
	5	7	8	54–66	6–7	275 (210–338)	220 (175–286)	−20 (−15 to −42)
	3	6	8	181–244	7	117 (105–140)	115 (105–131)	0
Adrenocortical Transplantation	15	9	12	114–186	60–77	136 (104–182)	136 (104–182)	0
Normal: restricted diet	4	6	7	36–57	7–8	343 (289–367)	240 (201–262)	−30 (−24 to −43)
	8	11	12	58–83	8	205 (175–254)	138 (87–192)	−33 (−15 to −55)
Normal: full diet after restricted diet	2	4	6	68–78	15	225 (192–306)	200 (157–254)	−11 (0 to −23)
Adrenalectomy: saline drinking water	10	21	18	32–52	5–11	336 (262–442)	340 (262–442)	0
	3	6	5	33–45	5–11	330 (245–442)	437 (350–520)	+32 (+21 to +42)
	2	4	5	47–49	14	291 (254–350)	418 (367–490)	+44 (+20 to +65)
	12	17	21	68–83	14–15	199 (166–227)	238 (201–280)	+20 (+15 to +52)
	6	9	11	104–172	21–28	103 (70–140)	142 (105–201)	+38 (+20 to +100)

postoperative week by adrenalectomized rats (−6 to −41 gm.) and their blank operated controls (+11 to +53 gm.). The degree of weight loss or gain, of course, depended on the age of the animals. In young growing rats losses were less and gains greater than in older rats approaching their maximum weight. The epiphyseal cartilages of the dieted group averaged around 30 per cent narrower than those of the controls, a narrowing somewhat greater than that found a week after adrenalectomy (Fig. 1, B). This change may be reversed, but not very promptly, by restoring the normal diet. After restriction of diet for 8 days, followed by a week with food *ad libitum*, the epiphyseal cartilages of 2 out of 6 rats did not differ significantly from that of their controls, but in the others the cartilages were still somewhat narrower (12 to 23 per cent), although not remarkably so.

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Adrenalectomy: saline drinking water (1–1½ weeks). Immediately after operation 23 adrenalectomized rats and 27 blank operated controls were given salt solution in place of drinking water. The adrenalectomized animals maintained their appetite and food intake, but 7 of them lost a little weight (not over 8 gm.) while the others did not gain as much as did the controls. From 5 to 11 days after operation there was no significant difference between the widths of the epiphyseal cartilages of 18 of the adrenalectomized rats and those of their controls. In the other 5 the cartilages were definitely wider (see Table 1). The saline drinking water had not affected the cartilage widths of the control rats for they were similar to those of untreated rats of comparable age.

Adrenalectomy: saline drinking water (2 to 4 weeks). A series of 26 adrenalectomized rats (22 males, 4 females) were given saline drinking water after 6 or 7 days on tap water and at the end of another 7 or 8 days their epiphyseal cartilages were compared with those of 21 litter mate controls (16 males, 5 females). In all, the cartilage widths were greater than those of the controls: around 15 per cent greater in 5 cases (probably not significant); around 20 per cent in 12 cases; between 30 and 40 per cent in 5; and 42, 52, 60, and 65 per cent respectively in the other four (Fig. 1, C; Table 1). Six other litters of older rats (see table 1) were placed on saline drinking water immediately after operation and their cartilages were measured 3 or 4 weeks after operation. At three weeks one adrenalectomized rat showed no difference from the controls, but three had cartilages which were 44, 64, and 64 per cent wider. At four weeks the increases were 20 per cent in one rat, from 30 to 40 per cent in four rats, and 50 and 100 per cent in two.

DISCUSSION

In untreated adrenalectomized rats the proximal epiphyseal cartilage of the tibia does suffer a reduction in width if the operation is performed during the early weeks of life (up to the 9th or 10th at least), but not if adrenalectomy is done after the period of rapid growth when equilibrium has been established in the tibia between proliferation of cartilage and endochondral ossification (see Ray, Evans and Becks, 1941). This might be because the older cartilage is less labile but changes in cartilage width in older rats do take place. This is shown by our results in salt treated adrenalectomized rats of 104–172 days of age and the experience of others with hypophysectomy and growth hormone. In fact, indications of an increase in sensitivity to growth hormone with advancing age (150 days) were found by Ray, Evans and Becks (1941), so it may be that the growth hormone 'released' by removal of the adrenals has just enough influence on the more sensitive older cartilage to offset the narrowing influence of untreated adrenal insufficiency.

A partial adrenocortical insufficiency such as that which causes adrenalectomized rats with cortical transplants to be more susceptible to stress although in good health, does not affect the width of the epiphyseal cartilage over periods of 2 or 3 months.

Since maintenance of appetite and a fair condition of nutrition by the administration of saline drinking water to adrenalectomized rats prevents the narrowing of their tibial cartilages, while a narrowing quite similar to that of the first post-operative week in untreated animals can be imitated in normal rats by restriction for a week of their diet to quantities consumed by adrenalectomized rats, it is concluded that the stunting of the cartilage after adrenalectomy is the result of inanition rather than a direct effect of adrenocortical insufficiency.

The condition of the cartilage may be restored to or towards normal in partially starved unoperated rats by resumption of a normal food intake, and to normal or beyond it (widening) in adrenalectoized rats by salt treatment which restores their appetite. The latter return to the normal condition more easily than do the starved animals although their food consumption may return to only about three-fourths normal. Possibly a 'release' of growth hormone from the pituitary due to absence of the adrenals stimulates the cartilage along with the reëstablishment of nutrition.

When adrenalectomized rats are protected from inanition by salt treatment for two weeks or more the tibial epiphyseal cartilage becomes wider than normal. This widening does not appear in the majority of cases until after the first post-operative week and a half, and is somewhat greater in older animals with presumably more sensitive cartilages (Ray, Evans and Becks, 1941). This may be taken as evidence for the extension of the 'end organ' hormone hypothesis mentioned in the introduction, i.e. a release of the growth ("chondrotrophic") hormone of the anterior pituitary gland, as well as of the corticotropic hormone, occurs near the end of the second week after adrenalectomy.

The possibility of a direct effect of the lack of adrenocortical hormones on metabolic processes must not be overlooked. Tepperman, Engel and Long (1943) have suggested that growth hormone and adrenocortical hormone have opposite effects on protein metabolism, and that removal of one gland results in relative hyperactivity of the other. Wells and Kendall (1940) showed that certain adrenocortical extracts inhibit the growth of normal rats, although they suggested that it was because of depression of pituitary activity by the adrenal hormones. Moreover, Becks et al. (1944) have shown that adrenocorticotropic hormone is antagonistic to growth hormone and retards chondrogenesis and osteogenesis in normal rats, but not in the absence of the adrenal glands.

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SUMMARY

During the first week and a half after adrenalectomy there is a reduction in the width of the proximal epiphyseal cartilage of the tibia in young growing rats, but not in fully grown rats. This may be prevented by salt treatment which restores appetite and food intake. It may be imitated in normal rats by restriction of the diet for a week. It is concluded that narrowing of the cartilage after adrenalectomy is the result of self imposed inanition.

Beginning near the end of the second week after adrenalectomy, in salt treated adrenalectomized rats, there is an increase in the width of the epiphyseal cartilage. Restoration of the cartilage occurs more readily upon salt treatment during the second week after adrenalectomy than upon restoring the diet after a week of food restriction. It is suggested that this may be accounted for by an increase ('release') in chondrotrophic activity on the part of the anterior pituitary gland.

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NOTES AND COMMENTS

METABOLISM OF THE STEROID HORMONES: THE EXCRETION OF ESTRONE AFTER THE ADMINISTRATION OF ESTRONE AND α-ESTRADIOL TO RHESUS MONKEYS[1]

METABOLITES of estrone and α-estradiol have been isolated from urine in various metabolism experiments involving men, guinea pigs, and rabbits. Guinea pigs, both ovariectomized females and normal males, have been shown to convert α-estradiol into estrone (Fish and Dorfman, 1940, 1941). In one series of experiments, when α-estradiol was administered to ovariectomized-hysterectomized rabbits, estrone and β-estradiol were isolated from the urine (Fish and Dorfman, 1942). Stroud (1939) administered estrone to normal rabbits and isolated estrone and an impure phenolic compound which he believed to be β-estradiol. Heard, Bauld, and Hoffman (1941) recovered estrone and β-estradiol from the urine of intact estrous and ovariectomized-hysterectomized rabbits after the administration of either estrone or α-estradiol. The conversion of α-estradiol to estrone (Heard and Hoffman, 1941) and of estrone to estriol (Pearlman and Pincus, 1943) has been reported in men.

We have studied the metabolism of both α-estradiol and estrone in ovariectomized-hysterectomized rhesus monkeys. In both instances estrone has been isolated and identified.

EXPERIMENTAL

Monkey A which had been ovariectomized and hysterectomized received 23 injections over a 25-day period of α-estradiol dipropionate.[2] Each injection consisted of 50 mg. of the hormone dissolved in 1 cc. of olive oil, making a total of 1150 mg. of α-estradiol dipropionate. Monkey B, a second avariectomized and hysterectomized monkey, was treated with estrone propionate. During a 27-day period the monkey received 25 injections of hormone. The hormone was administered in olive oil so that 1 cc. was equivalent to 50 mg. of hormone, making a total of 1250 mg. of estrone propionate.

The urines were collected under toluene and treated by simultaneous hydrolysis with hydrochloric acid and extraction with benzene. The residue from the benzene soluble portion was fractionated into the acidic, neutral, 0.1N sodium hydroxide soluble phenols, non-ketonic phenols not extracted from ether solution by 0.1N NaOH, and ketonic phenols not extracted from ether solution by 0.1N NaOH. The method of extraction and fractionation has been described previously (Fish and Dorfman, 1942).

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[2] We are indebted to Ciba Pharmaceutical Products, Inc. for the supply of hormone.

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The ketonic phenolic residues which were not extractable with 0.1N NaOH from an ether solution were dissolved in benzene and adsorbed on columns of aluminum oxide (Brockmann). Elutions were carried out with benzene, 1% ethanol in benzene, and 2% ethanol in benzene. The 1% ethanol in benzene eluates yielded estrone after recrystallization from ethanol and methanol. The details are presented in Table I. Estrone was isolated after the administration of both estrone and α-estradiol. The estrone was identified by its melting point, the melting point of the acetate, and melting points of mixtures of the free compound and its acetate with respective authentic samples. All melting points were taken with the Fischer-Johns apparatus and are uncorrected. From the estradiol experiments a yield of 36

TABLE I. RECOVERY OF ESTRONE AFTER THE ADMINISTRATION OF ESTRONE AND ESTRADIOL TO OVARIECTOMIZED-HYSTERECTOMIZED MONKEYS

Monkey	Substance administered	Amount administered	Time of administration	Weight of estrone isolated	M.P. of estrone[1] isolated	M.P. of acetate[2]
		mg.	*days*	*mg.*	° C.	° C.
A	α-Estradiol dipropionate	1150	25	36	249–252	121–122
B	Estrone propionate	1250	27	34	249–254	120–121

[1] The melting point of an authentic sample of estrone was 255–257° C.
[2] The melting point of an authentic sample of estrone acetate was 120–121° C.

mg. of estrone was realized, while the estrone experiment yielded 34 mg. of estrone. Thus recoveries of 4.4% and 3.3% in the form of estrone were realized for the estradiol and estrone experiments respectively.

SUMMARY

Estrone was isolated from the urine of ovariectomized-hysterectomized Rhesus monkeys which had received either α-estradiol dipropionate or estrone propionate. The yields of urinary estrone were 4.4% and 3.3% respectively after administration of 1150 mg. of α-estradiol dipropionate and 1250 mg. of estrone propionate.

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METABOLISM OF THE STEROID HORMONES: THE ISOLATION OF ISOANDROSTERONE AFTER THE ADMINISTRATION OF ANDROSTERONE

IN A PREVIOUS COMMUNICATION, we reported the isolation of isoandrosterone from urine after the administration of testosterone to normal male guinea pigs (Dorfman and Fish, 1940). Isoandrosterone has also been isolated from the urine of a hypogonadal male who received testosterone propionate (Dorfman, 1941), from the urine of a woman suffering from adrenal hyperplasia (Butler and Marrian, 1938), from the urine of a girl with polycystic ovaries (Hirschmann, 1941), and from normal women's urine and men's cancer urine, (Pearlman, 1940 and 1942). We now have evidence for the urinary excretion of isoandrosterone after the oral or subcutaneous administration of androsterone to normal male guinea pigs.

Experiment A

Three adult male guinea pigs received daily doses of 50 mg. of androsterone[1] orally in gelatin capsules for a period of three days. A total amount of 450 mg. of androsterone was administered. The animals were kept in metabolism cages during the period of hormone administration and for the following two days. The urine was simultaneously hydrolyzed with hydrochloric acid and extracted with benzene as previously described (Fish and Dorfman, 1942). The neutral fraction was separated into the ketonic and nonketonic fractions by means of the Girard-Sandulesco reagent T. Assays for androgenic activity indicated the equivalent of 400 I.U. in the ketonic neutral fraction. The ketonic neutral fraction was dissolved in 25 cc. of CCl_4 and adsorbed on 1 gram of aluminum oxide. (Brockman) elutions with 0.1% ethanol in CCl_4 yielded a white, crystalline residue which on recrystallizing from 75% methanol, 95% ethanol, and finally absolute methanol yielded 20 mg. of a substance melting at 173–174° C. A benzoate was formed which melted at 212–214° C. and when mixed with an authentic sample of isoandrosterone benzoate (M.P. 215–217° C.) the melting point was 214–216° C.

The mother liquors were combined, evaporated to dryness and crystallized from methanol. An additional amount (15 mg.) of isoandrosterone separated which melted at 167–168° C. After two recrystallizations from 95% ethanol, the melting point was 168–170° C. Acetylation yielded a compound which melted at 116° C. which did not depress the melting point of an authentic sample of isoandrosterone acetate.

Experiment B

Eighty milligrams of androsterone propionate, in 2 cc. sesame oil, was injected subcutaneously for three successive days into each of six adult male guinea pigs. A total of 1.44 gm. androsterone propionate or 1.2 gm. androsterone was administered.

The animals were kept in metabolism cages and all urine was collected

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[1] We are indebted to Ciba Pharmaceutical Products, Inc. for the supply of androsterone.

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under toluene throughout the injection period and for five days following the injections.

The urine was hydrolyzed by boiling under reflux for 10 minutes with 15% HCl, cooled, and extracted with benzene. Removal of acids and phenols yielded 529 mg. of neutral fraction which was found to contain 135 mg. of 17-ketosteroids, as determined by the method of Holtorff and Koch (1940).

After treatment of the neutral fraction with digitonin, 47 mg. digitonin precipitable material was obtained. Separation of this fraction into ketonic and non-ketonic material by means of the Girard-Sandulesco reagent T yielded 32.6 mg. of crystalline material in the ketonic fraction. After two recrystallizations from absolute methanol 22.5 mg. of a substance melting at 169–171° C. were obtained. When mixed with an authentic sample of isoandrosterone (M.P. 169.5–170° C.) the mixture melted at 170–170.5° C. An acetate was formed which melted at 95–96° C. This derivative is the lower-melting modification of isoandrosterone acetate. A mixture of this acetate with an authentic sample of isoandrosterone acetate (M.P. 114.5–115° C.) melted at 113–114° C.

A control sample of urine collected from six guinea pigs over an eight-day period, before the administration of androsterone, contained 35 mg. of 17-ketosteroids (0.73 mg. per day) in the total neutral fraction.

Whether the inversion of the C3 hydroxy group in androsterone is a result of a metabolic change or on the other hand a result of the method of hydrolysis employed cannot be decided at this time. If this metabolic change does take place, it presumably would proceed by oxidation to androstanedione-3,17 and subsequent reduction to isoandrosterone or by dehydration to Δ^2 or Δ^3-androstenone-17 with subsequent rehydration to isoandrosterone.

SUMMARY

Isoandrosterone was isolated from the urine of normal male guinea pigs after either the oral or subcutaneous administration of androsterone. Eight per cent of the orally administered androsterone and 2% of the subcutaneously administered androsterone was recovered as isoandrosterone.

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MODIFICATION OF THE ELECTROCHEMOGRAPH FOR GREATER ACCURACY AND SPECIFICITY IN THE ASSAY OF URINARY KETOSTEROIDS

Wolfe, Hershberg, and Fieser (1940) have described methods for the polarographic assay of the urinary ketosteroids. The authors have employed these techniques, in part, for the past 4 years in the routine assay of ketosteroids for clinical and research purposes. We have found the methods

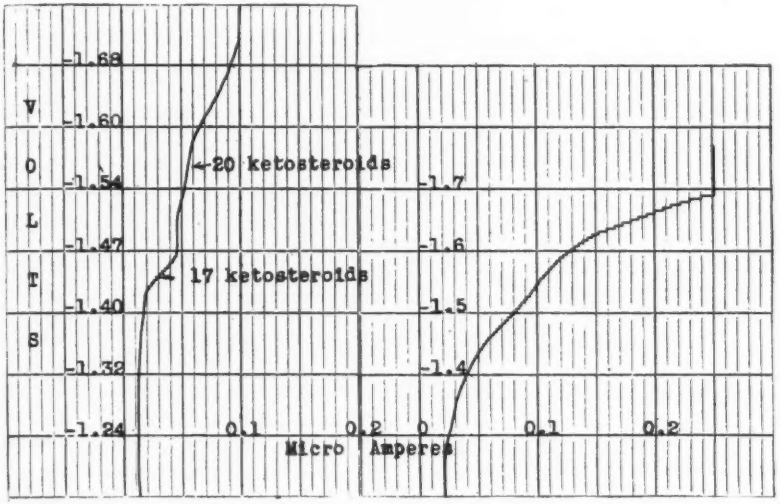

Fig. 1. The curves shown were both obtained on the same urine specimen with the apparatus set at the same sensitivity level. The curve at the left was drawn using the apparatus as modified by us. The curve at the right was obtained using the apparatus in its original form.

The current surges for the 17 and 20 ketosteroids are noted in the first curve. They are inseparable in the second.

ideally suited for this type of work provided the apparatus employed is slightly modified.

The apparatus used (Leeds and Northrup Electrochemograph) was a modification of the original type of recording polarograph. It employed a capacitance and resistance averaging circuit in the recording system. The purpose of the circuit was to reduce the oscillations in current inherent in the falling mercury drop method of polarographic analysis. Readings of the current flow through the circuit were made by the instrument every 2 seconds.

As constructed, therefore, a significant amount of time could elapse between a change in the rate of current flow in the polarizing cell and its recording by the meter. Furthermore, the recording was not a record of the

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rate of flow at the time the notation was made, but was really an average of the current flow for the last few seconds.

Such a situation would have been adequate for the polarography of those materials which have good ionic mobility or on mixtures of substances whose half wave potentials do not lie close together. But under the circumstances involved in the assay of urinary ketosteroids, the reciprocal situation held.

We found that if we reduced the rate of alteration of voltage that all our difficulties could be resolved. This reduction amounts essentially to giving the instrument sufficient time to record what has been going on in the polarizing cell. Instead of drawing the type of curve pictured in the earlier reports, the instrument would, under these conditions, draw curves which in many instances approximated in clarity those drawn for inorganic materials.

We found further that with the apparatus set up in this fashion that we could (1) utilize an electrochemical constant for our calculations and note solely the number of microamperes increment between the 2 ends of the curve. It was not found requisite to read the curves as described by the earlier workers. We could also (2) distinguish in our specimens the 20 ketosteroids from the 17 ketosteroids. The half wave potentials of these 2 groups are so close, -1.54 and 1.43 volts respectively, that they could not be separated by the instrument in its original form. This lack of distinction tended to give excess values for 17 ketosteroids when assays were made on urine specimens containing both types of material.

The reduction in rate of change of voltage was achieved simply by reducing the range of the potentiometer by 1 volt (from 0 to -3 to 0 to -2). The recording system and the automatically changing potentiometer were allowed to operate at the original speed. The net effect was to increase the speed of recording or number of recordings per polarographic analysis to 3/2 the original rate.

This change was expressed in the curves as drawn by an elongation of the ends of the curves by the factor, 1.5. This elongation of the slow change portion of the curve obviously made for more accurate determination of the beginning and end of the current surge due to the substance being studied.

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NEW BOOK

ENDOCRINOLOGY OF WOMAN. By E. C. Hamblen, B.S., M.D., F.A.C.S. i–xii, 1–571 pages, price: $8.00. Published by Charles C Thomas, Springfield, Illinois.

Endocrinology of Woman is a handbook which deals clearly, simply and accurately with those endocrine disorders which occur in the female of the species from birth through the climacteric. This volume is not a revised edition of the author's earlier *Endocrine Gynecology*, but is completely new in scope and format.

The first section of the book deals briefly with the endocrine glands and relates the physiological actions of each. In the second section the normal endocrine physiology of woman is considered with all of the detail necessary to an understanding of the factors involved. The chapters on adolescence and the climacteric are quite satisfactory, and are better than are usually found in manuals with so extensive a scope as this book. The chapters on the menstrual cycle and the anovulatory cycle are very similar to corresponding sections in the author's previous book, from which the illustrations of endometrial changes are copied. It is regrettable that new and more adequate microphotographs of the cyclic changes of the endometrium were not used in this volume.

The section on endocrine diagnostic methods is inclusive and detailed, and in general could serve as a laboratory handbook. The author quotes only one author on the "Xenopus Frog Test" for pregnancy diagnosis, and evidently does not speak from his own experience with the test. In contrast, the author describes the technique of pregnandiol assay, according to Venning, and reports adversely on the reliability of the test during the menstrual cycle, which is contrary to the experience of many laboratories.

The last and largest section of the book, entitled "Endocrinology Applied to Gynecological Disease" is quite inclusive. The reader finds here succinct paragraphs on adolescent acne, on obesity or on abnormal skeletal growth.

The chapter on dysmenorrhea does not cite all of the publications dealing with "cures" by means of endocrine preparations, but does give a survey of the problem which should be satisfactory to the internist or the general practitioner. Early in the chapter it is stated that "psychic factors play a major role in much functional dysmenorrhea," which statement sets the stage for consideration of endocrine therapy.

The chapters on sterility are among the most useful in the book, and the author draws heavily on his own extensive clinical experience in discussing endocrine therapy in this disorder.

In those sections usually considered of greater interest to general medicine as in chapter 29, "Pancreatic Diseases," the author deals quite adequately with those manifestations of diabetes which are of immediate interest to the gynecologist or the obstetrician.

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This book has a wealth of bibliographic references, appropriately placed at the end of each chapter. It is brief, accurate and simply written. It should be most useful to the medical student, and to the physician in general medicine who desires an explicit manual on the endocrine aspects of the diseases of women.

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